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EFFECTS OF SUB-MINIMAL GROWTH TEMPERATURES ON THE VIABILITY AND INJURY OF *STAPHYLOCOCCUS AUREUS*, *ESCHERICHIA COLI* AND *STREPTOCOCCUS FAECALIS*

by

© THOMAS EDWARD PATTERSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

IN

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DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled EFFECTS OF SUB-MINIMAL GROWTH TEMPERATURES ON THE VIABILITY AND INJURY OF STAPHYLOCOCCUS AUREUS, ESCHERICHIA COLI AND STREPTOCOCCUS FAECALIS, submitted by THOMAS EDWARD PATTERSON in partial fulfilment of the requirements for the degree of Master of Science in Food Microbiology.

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Date ....30 NOV 1973....

ABSTRACT

Staphylococcus aureus, Escherichia coli and Streptococcus faecalis were subjected to non freezing temperatures below their minimum growth temperatures to determine if this treatment would affect the isolation of these organisms on commercially available selective media.

Although no modification of the ability of Str. faecalis to grow on KF Streptococcus Agar could be demonstrated it was found that storage in this temperature range markedly reduced the ability of E. coli to grow on Violet Red Bile Agar and S. aureus to grow on Mannitol Salt Agar as compared to growth on a complex nutritional medium, Tryptic Soy Agar. Storage at low temperatures had little effect on the growth of S. aureus on Tellurite Polymyxin Egg Yolk Agar.

Exponential phase cultures were more sensitive to the storage temperatures than stationary phase cultures and the rapidity and extent of injury, as determined by sensitivity to selective media, were varied with pH of the injury menstruum. The rapidity and extent of injury to both S. aureus and E. coli increased with decreasing temperature from the minimal growth temperature to 0°C.

Both E. coli and S. aureus exhibited an extended lag period when incubated at 37°C after exposure to the experimental temperature range and both organisms recovered their tolerance to selective media during this lag period prior to the initiation of growth. E. coli recovered from injury at both pH 7.2 and 5.0 but injured cells of

S. aureus were sensitive to pH 5.0 and died off during the extended lag period prior to the growth of the uninjured cells.

The rapidity and extent of recovery of both E. coli and S. aureus was shown to vary with the incubation temperature but no recovery could be demonstrated after 24 h incubation at temperatures below the minimum growth temperatures.

This investigation emphasizes the need for careful screening and selection of media to be used for the isolation of organisms from foods held at refrigeration temperatures.

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INTRODUCTION

The metabolic activity of a microorganism is dependent upon a complex series of chemical and physiocochemical reactions all of which are influenced by temperature. The addition of energy, in the form of heat, to a chemical reaction causes an acceleration of the reaction rate over a wide range of temperatures, providing that the reactants are not heat labile. Arrhenius defined the correlation between temperature and the velocity of a chemical reaction by the following formula:

$$v = Ae^{-E/RT}$$

v = reaction velocity

A = entropy constant

E = activation energy

R = gas constant

T = absolute temperature

A plot of the natural logarithm of v against the reciprocal of the absolute temperature T results in a linear relationship.

Arrhenius later modified the equation when dealing with complicated biological systems by replacing E with μ, μ being referred to as the "temperature characteristic". In biological systems, because of heat lability of enzymes, the temperature range over which the Arrhenius equation holds true is much less than that of most chemical reactions.[2] Also in cellular systems a complex series of reactions is involved all of which have their individual temperature coefficients. Arrhenius' use of μ for biological systems is a recognition of this fact

and because of the complexity of biological systems the linear relationship between reaction rate and temperature only exists over a very narrow temperature range.

All microorganisms exhibit a strict range of temperature over which they will grow and a temperature or very narrow temperature range at which they grow optimally. In other words, all microorganisms possess a minimum, optimum and maximum growth temperature.

The minimum and maximum growth temperatures can be defined as the temperatures at which the number of viable cells present in a population remain practically constant i.e., the temperature at which there is an equilibrium between the death and multiplication rate of the cells in the entire population (Schmidt-Lorenz 1967). For practical purposes however, the maximum and minimum growth temperatures are those at which even the slightest increase in the number of viable cells is detectable. It should be recognized that any determination of temperature limits for growth is necessarily subjective. The incubation time, cultural conditions and means of assessing growth are arbitrarily selected by the observer. These factors assume particular significance in determination of minimum growth temperatures where the lag period of the organism may be markedly extended. A consequence of this is the increased possibility of contamination and failure of equipment.

The optimum growth temperature for a microorganism is defined by Ingraham (1962) as that temperature at which the specific growth rate is maximal. The specific growth rate (k) can be calculated from the equation:

$$k = \frac{2.303 (\log x_2 - \log x_1)}{t_2 - t_1}$$

x_2 and x_1 are any measure of cellular activity for example, cell numbers at times t_2 and t_1 at a stage when the culture is in a state of balanced growth.

The fact that all microorganisms exhibit a minimum, optimum and maximum growth temperature and that these temperature criteria vary between organisms has been used to separate microorganisms into three main groups, psychrophiles, mesophiles and thermophiles. All three temperature criteria have been used in formulating these divisions but it has often been found more practical and precise to use only one parameter to ascertain the classification.

Psychrophiles for example have been defined as having an optimum growth temperature of less than 15°C (Burrows 1959) and as having a maximum growth temperature of 30°C (Stanier 1970). The most accepted definition of a psychrophile however is that of Stokes (1963) who used the minimum growth temperature as a parameter. He defined a psychrophile as an organism capable of producing microscopically visible colonies on a solid medium within one week at 0°C. Farrell and Rose (1967), further divided the psychrophiles into obligate and facultative psychrophiles according to their optimum growth temperature. Psychrophiles with an optimum growth temperature of greater than 20°C were termed facultative psychrophiles while those with an optimum temperature of less than 20°C were termed obligate psychrophiles.

Mesophiles are generally characterized by their growth optima which lie between 25° and 40°C while thermophiles are defined as having a maximum growth temperature above 50°C (Farrell and Rose 1967). Thermophiles can be further subdivided into obligate and facultative

thermophiles. An obligate thermophile has an optimum growth temperature between 65° - 70°C and a minimum of 40° - 42°C while a facultative thermophile has a maximum growth temperature between 50° - 65°C and a minimum of room temperature or less (Farrell and Campbell 1969).

In food preservation and processing temperature plays a vital role. Heat sterilization, freeze drying, freezing and low temperature storage are in widespread use, therefore it is vital for the food microbiologist to gain an understanding of the physiological or biochemical factors that determine the temperature limits for growth and survival of microorganisms and to assess the damage to the microbial cell from exposure to temperatures outside its normal growth range. Sterilization has been extensively examined and in view of this the following discussion will be limited to the effects of sub-lethal heating, freezing and thawing and chilling.

Sub-Lethal Heating

Pasteur (1876) stated that yeasts heated in beer could withstand a temperature of 55°C without losing the capacity to germinate but the action was rendered somewhat more difficult and slower. This is the first reported observation on sub-lethal heat treatment. Since that time the effects of sub-lethal heat treatment on a wide variety of organisms have been extensively investigated. The incubation period allowed for the growth of heat treated bacteria in tests of survival was shown to be an important factor by Isaacs (1930a), as was the pH of the recovery medium (Isaacs 1930b). Fay (1934), Curran and Evans (1937) and Nelson (1940, 1942, 1943a,b), all found that microorganisms subjected to sub-lethal heat treatment showed greatly increased survival on enriched

recovery media.

Jackson and Woodbine (1963), showed that when an enterotoxigenic strain of Staphylococcus aureus was subjected to sub-lethal heat treatment and subsequently inoculated into nutrient broth at 37°C there was a drop in viable count followed by a lag phase of growth. The phenomenon was described as an extended lag phase. More recent work has shown that the extended lag phase is in actuality a recovery period. Busta and Jezeski (1963) reported that when staphylococci were subjected to sub-lethal heat treatment the survivor count on plate count agar was greater than on Staphylococcus Medium 110, i.e., the cells were rendered salt sensitive. Stiles and Witter (1965) investigated the growth of S. aureus after heat treatment. They found a complete nutritional media, Trypticase Soy Agar, to be far superior to Trypticase Soy Agar + 7.5% NaCl, Chapman Stone Medium, Staphylococcus Medium 110, Mannitol Salt Agar, Azide Blood Agar Base and Tellurite Glycine Agar for supporting growth of the heat treated organism.

Iandolo and Ordal (1966), confirmed the results of Busta and Jezeski by demonstrating that injured Staphylococcus aureus was unable to reproduce on media containing 7.5% sodium chloride but could recover their reproductive ability after incubation in a medium containing an energy source, a complex nitrogen source and phosphate. Both energy source and nitrogen source requirements were modified by the injury as neither NH_4Cl, mannitol, ribose, trisodium citrate or galactose would support recovery. They also illustrated that recovery was dependent on pH and temperature of incubation, the minimum recovery temperature being between 3 - 7°C and the minimum pH being between 4 - 5. The optimum pH

for recovery was found to be 7.2, which conflicts with an optimum pH of 6.0 reported by Allwood and Russell (1968). They also provided data to show that nucleic acid resynthesis was an important aspect of the recovery process. Sogin and Ordal (1967), heated S. aureus at 55°C for 15 min and found that the organisms exhibited an extended lag period during which the organisms regained their ability to grow on 7.5% NaCl agar. By the use of metabolic inhibitors and antibiotics they found that protein synthesis was not involved in the recovery process however, nucleic acid synthesis was shown to be required. The primary nucleic acid species involved was shown to be rRNA. The rRNA was degraded during heat treatment and resynthesized during recovery.

Clark et al. (1968) found the recovery of heat injured Streptococcus faecalis to be much slower in a synthetic medium than on a complex one. They found that most of the commercially available enrichment media would support the recovery of the organism but at a slower rate. The injured cells became more sensitive to incubation temperature and pH. They also demonstrated that RNA synthesis and ATP synthesis were essential for recovery of the organism but protein synthesis was not involved.

Clark and Ordal (1969) investigated the effect of sub-lethal heat upon the growth of Salmonella typhimurium on several commercially available selective media. They found injured organisms to be sensitive to Brilliant Green Agar, Levine Eosin Methylene Blue Agar, Salmonella Shigella Agar and Desoxycholate Citrate Agar. The organism recovered its ability to grow on these media after incubation in Trypticase Soy Broth. Tomlins and Ordal (1971), showed that ATP synthesis, RNA

synthesis and protein synthesis were essential for the recovery of Sal. typhimurium from injury induced by sub-lethal heat. Gray et al. (1973), characterized the effects of thermal stress on Pseudomonas fluorescens. They confirmed the results of Allwood and Russell (1967), by demonstrating leakage of 260 nm absorbing materials from the cell, an indication of both membrane damage and thermally induced RNA degradation. Recovery was found to be dependent upon RNA synthesis. Tomlins et al. (1971), also demonstrated that sub-lethal heating inactivated key tricarboxyllic acid cycle enzymes in both Staphylococcus aureus and Salmonella typhimurium.

Hurst et al. (1973) confirmed earlier findings that S. aureus heated at 52°C lost their tolerance to sodium chloride. Salt tolerance was regained on incubation in a complex medium or in a diluted dialyzed medium in which unheated cells were unable to grow. Heat injury caused 30% loss of lipid and during the recovery period the concentration of C_{15} and C_{17} fatty acids returned to normal and there appeared to be an oversynthesis of C_{16} and C_{18} unsaturated acids. They also found that the K/Na ratio was 12.6 in control cells and 3.4 in injured cells. The K/Na ratio remained fairly constant during the recovery of salt tolerance. Thus it would appear that heat damaged cells of S. aureus may recover their salt tolerance while various membrane functions remain impaired. These findings introduce a new dimension into existing concepts of heat injury in microorganisms.

Chilling and Freezing

Although little work has been done on the viability of mesophiles held at subminimal growth temperatures but above freezing, many

investigations have been carried out on the effect of sudden chilling upon the viability of mesophiles. Sherman and Albus (1923) demonstrated that sudden chilling of a dilute suspension of Escherichia coli from its normal growth temperature to near 0°C caused a loss in viability. This phenomenon has since been termed "cold shock", and is confined mainly to gram negative organisms. "Cold shock" has since been shown to occur with other strains of E. coli (Sherman and Cameron 1934, Meynell 1958) and a drop in survival of as much as 10,000 fold has been noted. Strange and Dark (1962), and Strange and Ness (1963), demonstrated shock in Aerobacter aerogenes and Serratia marcescens. They showed that "cold shock" was accompanied by the release of ultraviolet absorbing materials, amino acids and ATP, indicating a form of damage to the cell's permeability barrier. Gorill and McNeil (1960) observed "cold shock" in Pseudomonas pyocyanea and Salmonella typhimurium but failed to induce shock in S. aureus. They found that only organisms in the exponential phase of growth were susceptable to "cold shock" and that the occurrence of shock was influenced by the concentration of cells, the growth medium, the suspending medium and the rapidity of chilling. These observations were further supported by Strange (1964). Strange and Dark (1962) reported that sucrose, Mg^{++}, Ca^{++} and bacteria free filtrates from chilled concentrated suspensions of exponential phase cultures protected A. aerogenes from "cold shock". The protective effect of divalent cations was confirmed by Farrell and Rose (1968) working with Pseudomonas fluorescens and by Sato et al. (1968) using germinating Bacillus subtilis spores. Sato and Takahashi (1969) demonstrated that E. coli, P. fluorescens and B. subtilis were susceptable to shock when in the

exponential phase of growth and that the viability of cold shocked cells increased rapidly when incubated at 30°C with suitable additives. They further demonstrated that the recovery would not occur in the absence of Mg^{++} and ATP generation.

The effects of freezing and thawing upon the microbial cell have been extensively investigated over a period of almost a century. Pictet and Young (1884) exposed Saccharomyces cerevisiae to temperatures of -70°C for 108 hours and -130°C for a further 20 hours and found that the yeast lost its ability to raise bread. Since then much work has been carried out in an effort to elucidate actual damage to the cell caused by freezing and thawing and to determine the external manifestations of this damage. Populations of coliforms and salmonella in foods were found to be progressively less able to grow on selective media as compared to non-selective media after storage at subzero temperatures (Gunderson and Rose 1948, Hartsell 1951). Straka and Stokes (1959) noted that 3 strains of Pseudomonas and one of E. coli gave similar colony counts on Trypticase Soy Agar and a minimal salts agar but after freezing and thawing the counts on the minimal agar were much lower than on Trypticase Soy Agar. Similar results were reported for Shigella sonnei (Nakamura and Dawson 1962) and for Pseudomonas fluorescens and E. coli (Arpai 1962).

By this time it had become apparent that any measurement of the survival of cells exposed to freezing was dependent upon the medium used to assay viability. Those bacteria capable of growth on a non-selective and highly nutritious medium but not on a selective or minimal medium were termed "metabolically injured". From this point efforts were directed towards defining the nutritional modifications induced by

freezing and thawing and assessing cellular damage which could influence these alterations. Moss and Speck (1966a) showed that freezing affected the cell membrane as evidenced by leakage of RNA nucleotides and short chain peptides from E. coli subjected to freezing. They reported that the peptide fraction offered protection against the effects of freezing when added to the freezing menstruum of a fresh culture prior to freezing. Moss and Speck (1966b) identified short chain peptides in trypticase that were instrumental in promoting growth of the injured cells, thus confirming the report of Straka and Stokes (1959), that freezing caused certain cells of a bacterial population to lose their ability to use inorganic nitrogen. MacLeod et al. (1966, 1967) demonstrated leakage of cellular components from Aerobacter aerogenes and E. coli subjected to chilling and lyophilization and showed that the cells became increasingly permeable to extra-cellular solutes. They presented evidence suggesting that trace amounts of toxic ions can penetrate the cytoplasmic membrane after freezing and thawing and that compounds in complex media permitted growth of the injured cell by chelating the toxic ions. An extended lag period similar to that caused by sub-lethal heating has been shown to occur following freezing and thawing (Postgate and Hunter 1963). Arpai (1963) suggested that the extended lag period was in reality a recovery period during which damage to the cell was repaired. Ray and Speck (1972a) found that Escherichia coli gave maximum recovery from injury resulting from freezing when incubated in a complex medium such as trypticase soy broth but demonstrated that the addition of inorganic phosphate and magnesium sulphate to a minimal medium enhanced the rapidity of repair. They reported recovery at pH

values from 4 - 10 with an optimum between 8 - 9. The minimum recovery temperature was above 15°C with a maximum between 25 - 35°C. The injured cells were found to be extremely sensitive to surface active agents common in selective media, sodium deoxycholate and sodium lauryl sulphate, as well as to lysozyme.

By the use of selective inhibitors Ray and Speck (1972b) demonstrated repair of E. coli in the absence of DNA synthesis, protein synthesis, RNA synthesis and mucopeptide synthesis but found ATP synthesis to be essential.

Many of the characteristics of organisms injured by heating and freezing, namely 'extended lag recovery period', leakage of cellular material, increased sensitivity to selective media and increased nutritional requirements result from freeze drying (Sinskey and Silverman 1970; Ray et al. 1971; Ray et al. 1972).

From this review of the literature it is apparent that sub-lethal heating, freezing and thawing and freeze drying can cause sufficient metabolic injury to microorganisms to render them sensitive to many common selective media. There is dearth of information however, on the effect of storing bacteria at temperatures below their minimum growth temperature but above freezing. In this temperature range, while cell division does not occur many metabolic processes within the cell are operational. For example, Goldstein et al. (1964) demonstrated protein synthesis in E. coli at 0°C while Friedman et al. (1971) reported RNA and DNA synthesis in E. coli at 7°C. The continuance of these metabolic activities at restrictive temperatures could result in cells with abnormal physiological characteristics. Could these abnormal

characteristics affect isolation and identification of the microorganisms in a manner analogous to other conditions of thermal stress? As most of the important pathogens and indicator organisms associated with foods are mesophilic, commercial refrigeration temperatures (0 - 10°C) represent such restrictive conditions for this group of microorganisms. An investigation of this problem would appear to be justified in view of the widespread and ever increasing use of refrigeration as a means of food preservation. Since many food products are preserved by storage in this temperature range it is of extreme importance to determine whether exposure to these temperatures could cause metabolic damage to food borne bacteria that would affect their enumeration on selective media. For the purposes of this investigation the organisms E. coli, Str. faecalis and S. aureus were chosen because they are important indices of food sanitation and many of the recommended methods for their enumeration do not involve pre-enrichment techniques.

MATERIALS AND METHODS

Test Organisms and Growth Media

Staphylococcus aureus NCTC 8532, *Escherichia coli* and *Streptococcus faecalis* were used throughout the investigation.

Stock cultures were maintained at 0°C on Tryptic Soy Agar slants and subcultured monthly.

The complex media used for all organisms in the experiments were Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA).

Selective media used were: Mannitol Salt Agar (MSA) and Tellurite Polymyxin Egg Yolk Agar (TPEY) for *S. aureus*, Violet Red Bile Agar (VRBA) for *E. coli*, and KF Streptococcus Agar (KFA) for *Str. faecalis*.

All media were supplied by Difco Laboratories Ltd., Detroit 1, Michigan, U.S.A. and all were sterilized as recommended by the manufacturer.

Preparation of Inoculum

Erlenmeyer flasks (250 ml) containing 100 ml aliquots of TSB were inoculated with a loopful of the test organism from the stock agar slants. The flasks were incubated in a shaking incubator (150 rpm) at 37°C (Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.). Aliquots from the cultures at the exponential phase (absorbance @ 600 nm .45 - .50) were transferred to new flasks of sterile pretempered TSB. Three such subcultures were made before the final sub-

culture, which was allowed to develop to the required phase of growth used for inoculation, either exponential phase (absorbance .45 - .50) or stationary phase (18 h culture).

Viable Count

Dilutions of the TSB cultures were prepared by transferring 1 ml aliquots to screw capped test tubes containing 9 ml (±0.2 ml) sterile peptone water (distilled water containing 0.1% Bacto peptone, pH 7.2). The dilutions were agitated on a vortex mixer for thirty seconds and serial dilutions prepared by transferring further 1 ml aliquots to other peptone water dilution blanks. Appropriate dilutions were then plated in triplicate on the complex medium (TSA) and the appropriate selective medium. The plating method used was a surface spread plate method described by Harrigan and McCance (1966). In this method sterile calibrated Pasteur pipettes are used to deliver uniform drops onto the surface of pre-poured dried plates of sterile media. The drops of diluted sample are then spread uniformly over the surface of the agar with the aid of a sterile spreader. The Pasteur pipettes used were calibrated to deliver 33 drops/ml.

Plates of media were prepared by pouring approximately 10 ml sterile media into petri dishes in a Laminar flow hood (The Baker Co., Inc., Sanford, Maine, U.S.A.). The covers were left off the petri dishes for 15 min while the plates were still in the hood to ensure that the surface of the plates would be adequately dry before use. Poured petri dishes of media were stored in plastic bags in a cold room at 4°C but were always used within 2 weeks after preparation.

Inoculated spread plates were incubated at 37°C in a hot air incubator (Precision Scientific Co., Chicago, Illinois, U.S.A.) for the recommended period of time (24 h for TSA and VRBA, 48 h for MSA and TPEY and 72 h for KFA).

The colonies formed after incubation were counted with the aid of a darkfield colony counter and the number of colony forming units in the original sample was calculated.

Conditions of Injury

Tryptic soy broth was used as the suspending menstruum in all cases. The pH of the TSB was adjusted with 0.1 N hydrochloric acid as required and sodium chloride was added to the broth formulation to attain the desired concentrations in the investigation of the effect of sodium chloride on extent of injury.

A 0.1% inoculation level was used for cultures exposed to injury at the stationary phase while a 1.0% inoculation level was used for cultures in the exponential phase to give an initial cell concentration of approximately 10^6 cells/ml. All organisms were injured by inoculation into sterile TSB pretempered to the desired temperature and subsequently stored at that temperature, with enumeration at appropriate intervals.

Two methods were used to attain the desired injury temperatures. The first method consisted of inoculating 100 ml quantities of TSB in 250 ml Erlenmeyer flasks with the appropriate concentration of organisms. The desired temperature was achieved by storage in a Metabolyte Refrigerated Water Bath Shaker (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, U.S.A.) shaking at 150 rpm. The accuracy of the

temperature control of this unit is ±0.2°C.

The second method consisted of inoculating 1000 ml quantities of TSB in 2 l side arm Erlenmeyer flasks containing Teflon coated magnetic stirring bars. Prior to inoculation the flask of medium was placed in a 12" x 12" x 12" plexiglass tank fitted with a coolant circulating device that maintained the level of coolant in the plexiglass tank above the level of medium in the flask. The coolant was stored in a separate water bath fitted with a heating and refrigeration system. The temperature of the coolant in the bath was controlled by a micro-set thermoregulator (Precision Scientific, Chicago, Illinois, U.S.A.) with a sensitivity of ±0.01°C.

Agitation of the medium in the flask was achieved by the use of a non-heating magnetic stirrer (Bellco Glass Inc., Vineland, New Jersey, U.S.A.).

Injury was determined by the difference in viable count on a complex medium (TSA) and the count on a selective medium for the particular test organism.

Recovery Conditions

Recovery of tolerance to selective media was studied in the following manner. Erlenmeyer flasks (250 ml) containing 100 ml of test culture previously exposed to injurious conditions were transferred to a Metabolyte shaking water bath at 37°C, agitating at 150 rpm. The viable count was determined by surface plating on both the complex medium (TSA) and the selective medium at appropriate time intervals. Growth rates of control cultures were also determined by incubating inoculated 100 ml aliquots of TSB in 250 ml Erlenmeyer flasks in the Metabolyte bath at

37°C at 150 rpm and enumerating at appropriate intervals.

Temperature Gradient Incubator

A temperature gradient incubator (Packer et al. 1973) was used to study temperature effects on the growth, injury and recovery of the organisms. This apparatus consists of an aluminum block into which holes have been drilled to accommodate culture tubes. Each end of the block is connected to a temperature control unit. By adjusting the temperature of the two ends it is possible to establish any particular temperature gradient along the block. For studies of growth and injury Astell roll tubes containing 15 ml of sterile TSB were inoculated with a 1% inoculum of exponential culture of the organism. The tubes were incubated in the temperature gradient incubator over a range of 0 - 11.5° C, and plate counts were performed on selective and complex media at appropriate intervals. The temperatures to which the organisms were exposed by this method were 0, 1, 2.5, 3.5, 5.5, 6.5, 8.0, 9.0, 10.5 and 11.5°C.

For studies on recovery a 1000 ml exponential culture of the organism in TSB was injured as described previously and the extent of injury determined by plating on selective and complex media. Fifteen ml aliquots of the injured culture were dispensed into sterile Astell roll tubes and incubated in the temperature gradient incubator over a range of 5 - 20°C for 24 h. Extent of recovery of the organism was then determined by enumeration on the appropriate selective media and TSA.

Throughout the course of the investigation, all experiments were repeated at least twice.

RESULTS

Metabolic Injury

Injury to Stationary Cultures

Erlenmeyer flasks (250 ml) containing 100 ml TSB, pretempered to 4°C in a Metabolyte shaker bath, were inoculated at the 0.1% level from a 18 h TSB culture of the test organism. Aliquots from the same culture were used to inoculate the TSB at pH 7.2 and 5.0 and the flasks were shaken at 150 rpm during storage.

Injury to S. aureus was demonstrated at both pH 5.0 and 7.2 as shown in Figs. 1 and 2. A difference of 1 1/2 log cycles between viable counts on TSA and MSA after 30 days storage at 4°C, pH 7.2, is shown in Fig. 1. At pH 5.0 the extent of injury was less than at pH 7.2 (Fig. 2). No injury was found to occur to an 18 h culture of E. coli after storage for 30 days at 4°C at either pH 7.2 or 5.0. In other words, no marked difference in count between the complete nutritional medium TSA and the selective medium VRBA could be detected after storage (Figs. 3 and 4).

Because E. coli showed no injury at 4°C and the strain of Str. faecalis grew at 4°C the experiment was repeated with inocula of 18 h cultures of both organisms at a storage temperature of 1°C. Under these conditions no significant differences in count on the complete nutritional media and the selective media could be demonstrated at either pH after 34 days storage (Figs. 5 - 8).

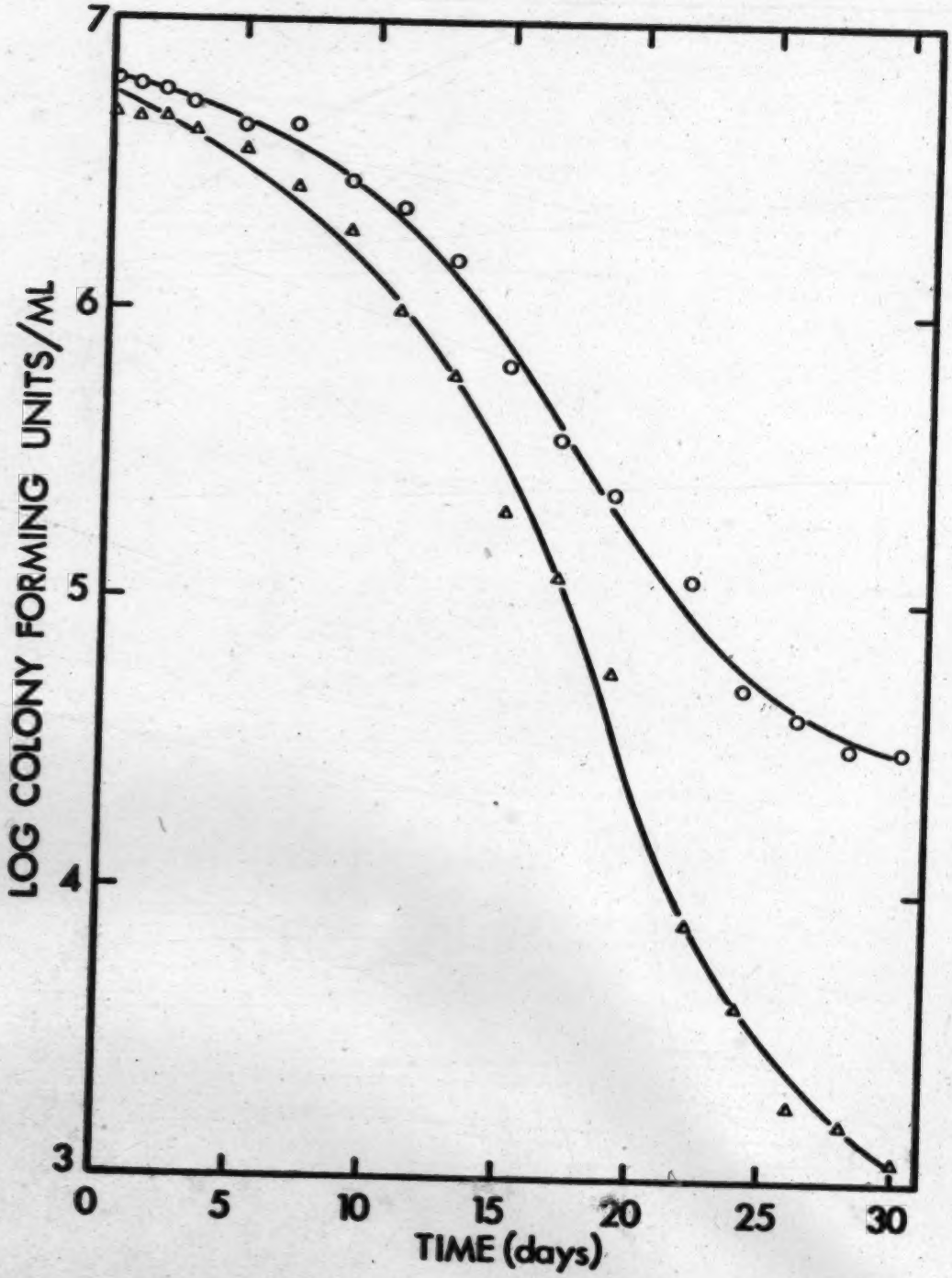

Fig. 1. Effect of storage at 4°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 7.2). Plating media ○ – TSA; △ – MSA.

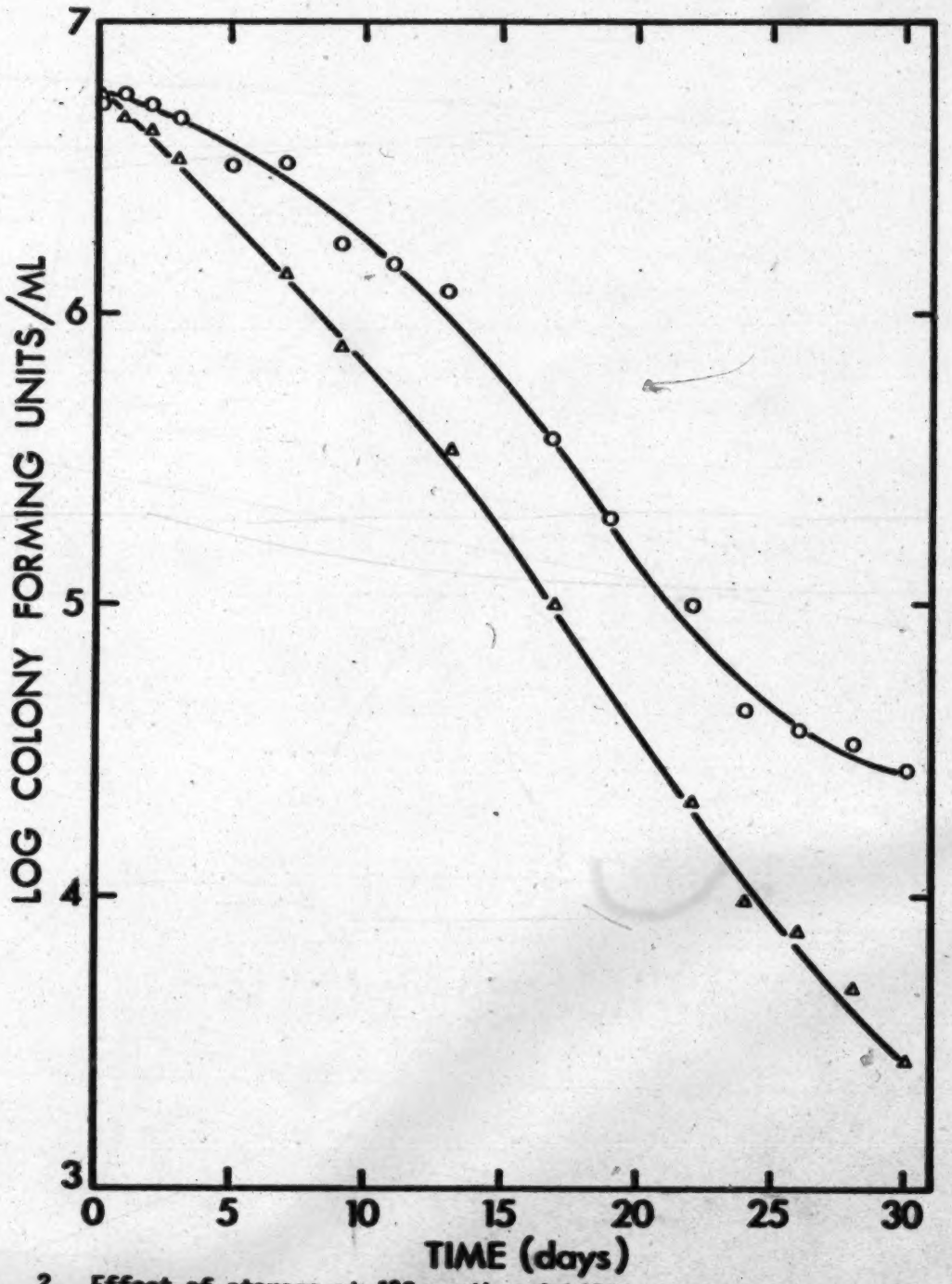

Fig. 2. Effect of storage at 4°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 5.0). Plating media o — TSA; Δ — MSA.

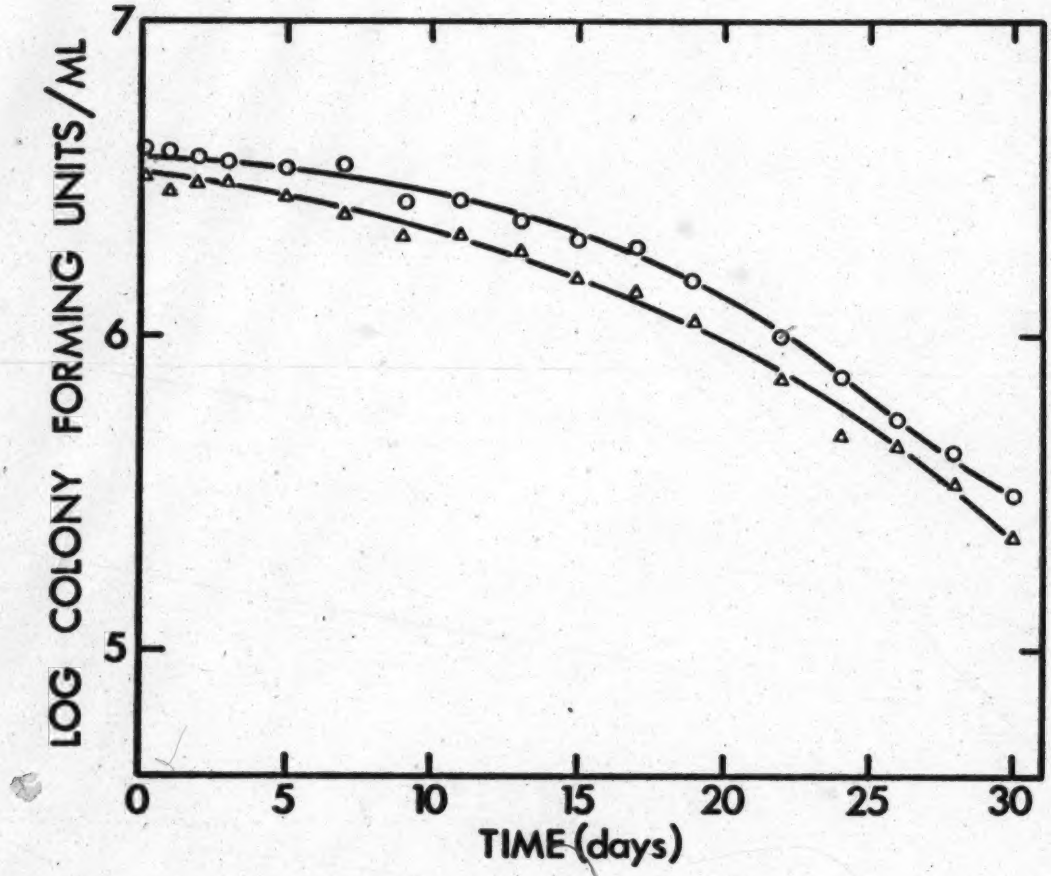

Fig. 3. Effect of storage at 4°C on the viability of a stationary phase inoculum of E. coli in TSB (pH 7.2). Plating media ○ — TSA; △ — VRBA.

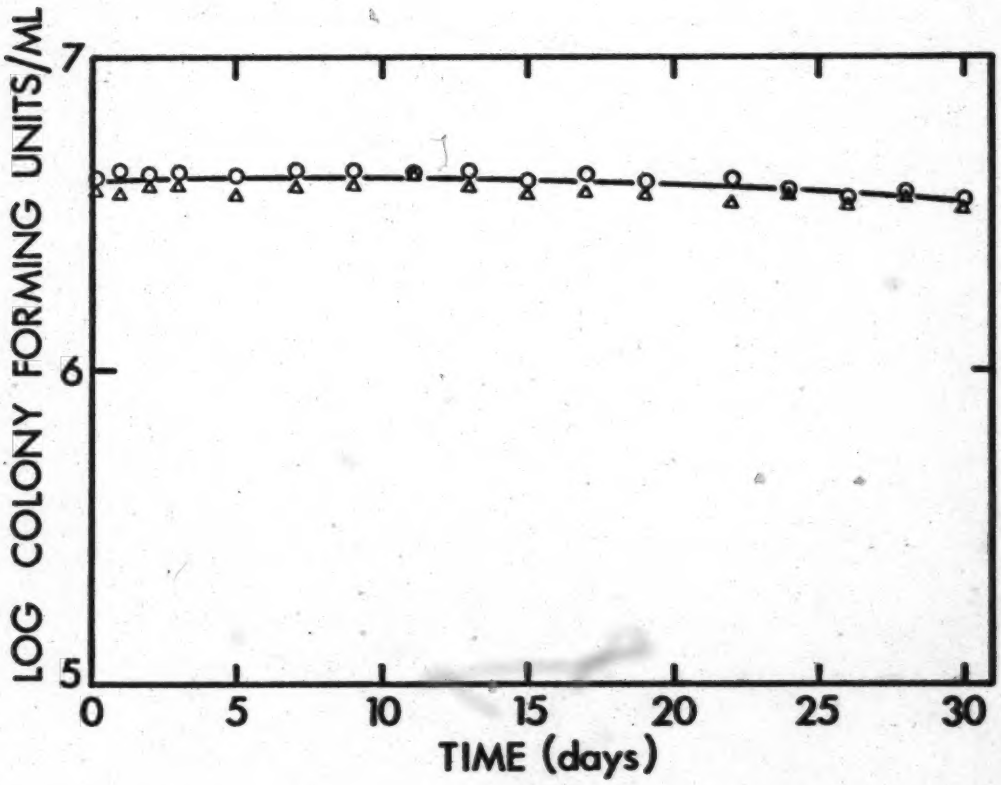

Fig. 4. Effect of storage at 4°C on the viability of a stationary phase inoculum of E. coli in TSB (pH 5.0). Plating media o—TSA; Δ—VRBA.

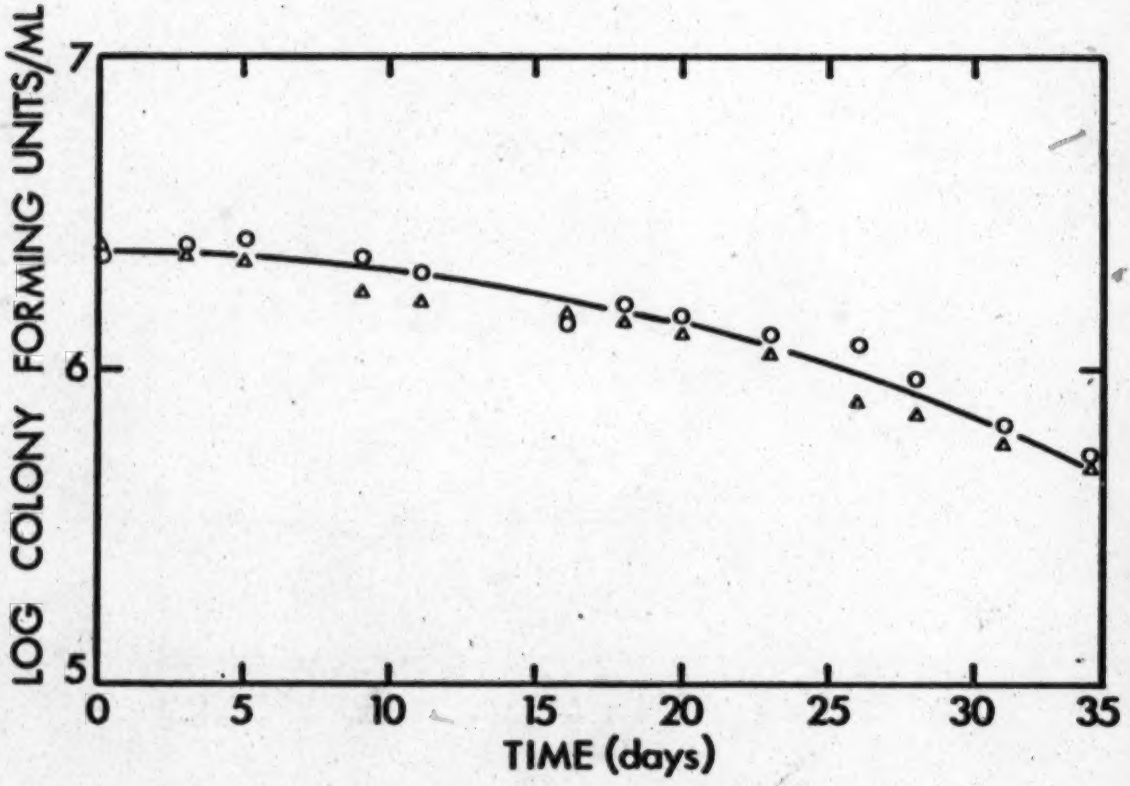

Fig. 5. Effect of storage at 1°C on the viability of a stationary phase inoculum of E. coli in TSB (pH 7.2). Plating media ○—TSA; △—VRBA.

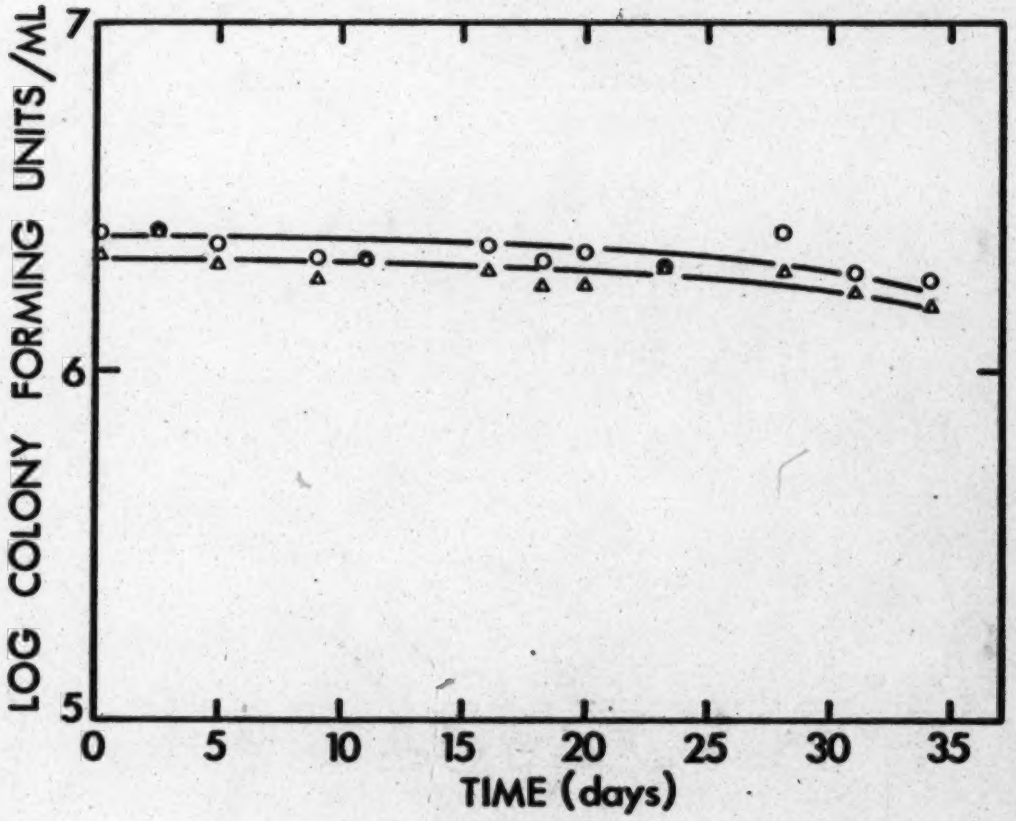

Fig. 6. Effect of storage at 1°C on the viability of a stationary phase inoculum of E. coli in TSB (pH 5.0). Plating media o—TSA; Δ—VRBA.

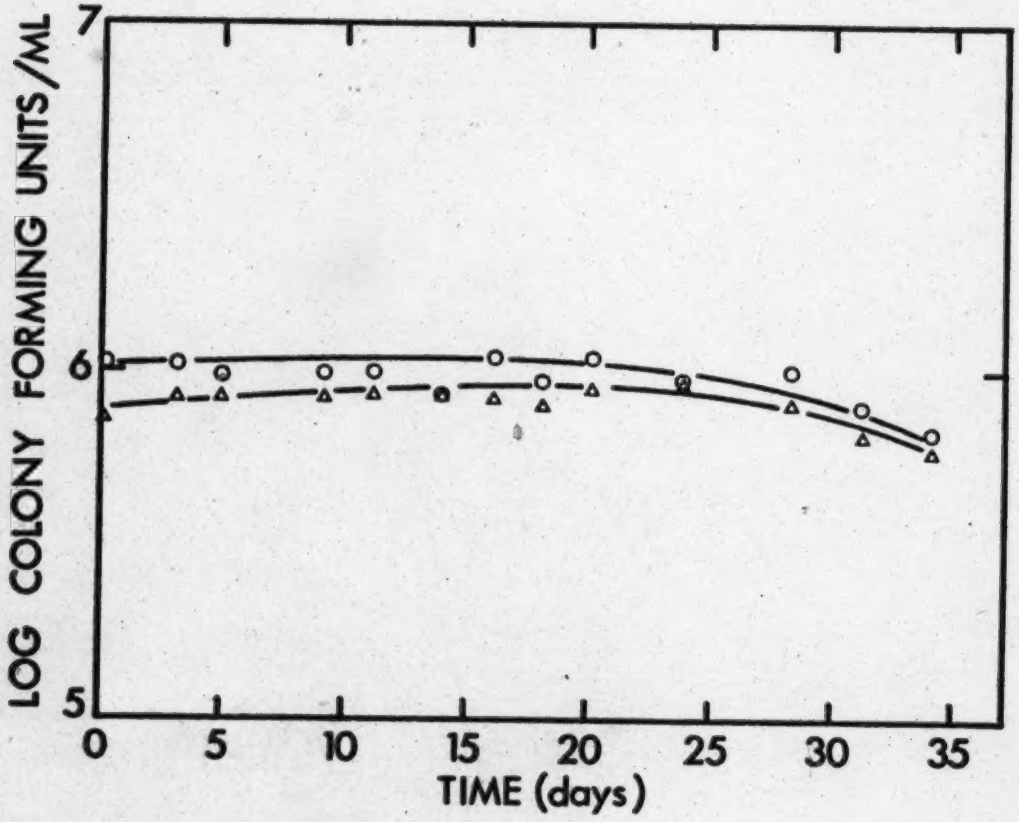

Fig. 7. Effect of storage at 1°C on the viability of a stationary phase inoculum of Str. faecalis in TSB (pH 7.2). Plating media ○—TSA; △—KFA.

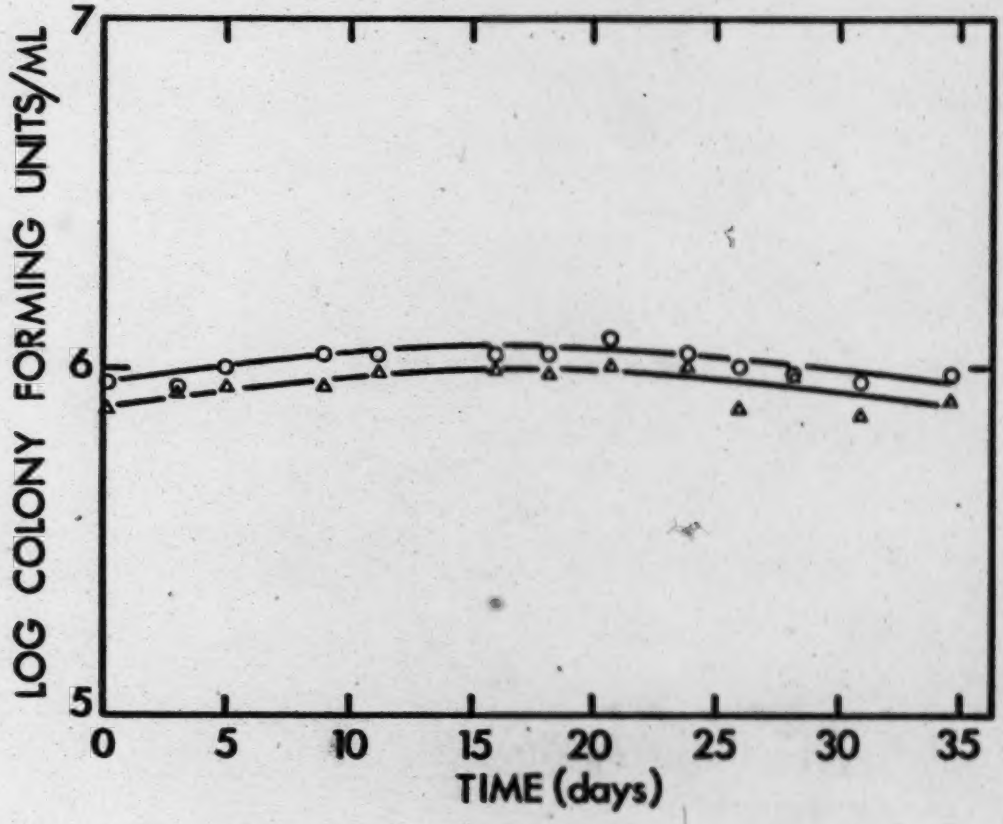

Fig. 8. Effect of storage at 1°C on the viability of a stationary phase inoculum of Str. faecalis in TSB (pH 5.0). Plating media o—TSA; Δ —KFA.

Effect of NaCl in injury menstruum

The effect of increasing salt concentration (NaCl) in the storage medium (TSB) on the extent of injury to S. aureus was investigated. Tryptic Soy Broth (0.5% NaCl) was supplemented with NaCl to give final salt concentrations of 3, 5 and 10%. 100 ml aliquots of these media were inoculated with a 0.1% inoculum of an 18 h culture and stored in a Metabolyte bath at 4°C as previously described. Unsupplemented TSB at pH 7.2 and 5.0 were inoculated and stored as controls. All flasks were inoculated from the same 18 h culture. Viable counts were made on TSA, MSA and TPEY agars. The results are shown in Figs. 9 - 13. A comparison of Figs. 9 and 10 with Figs. 1 and 2 illustrates the variability of the 18 h inoculum with respect to extent and rapidity of injury even though the inoculum was produced under standardized conditions. Injury was demonstrated by a difference in count on TSA and MSA under all conditions. However very little difference in count was observed between TSA and TPEY. By comparing Figs. 11, 12 and 13 with Fig. 9, it may be seen that increasing salt concentration in the injury medium seems to have little or no effect upon the extent of injury although at higher salt concentrations injury appeared to occur somewhat less rapidly.

Effect of temperature on injury

To study the effect of temperature on the injury of S. aureus the plexiglass baths containing side arm flasks with 1 l of TSB (pH 7.2) were used. A 0.1% inoculum of an 18 h culture was added as previously described. It can be seen from Figs. 14 - 17 that loss of viability (decrease in the count on TSA) and extent and rapidity of injury

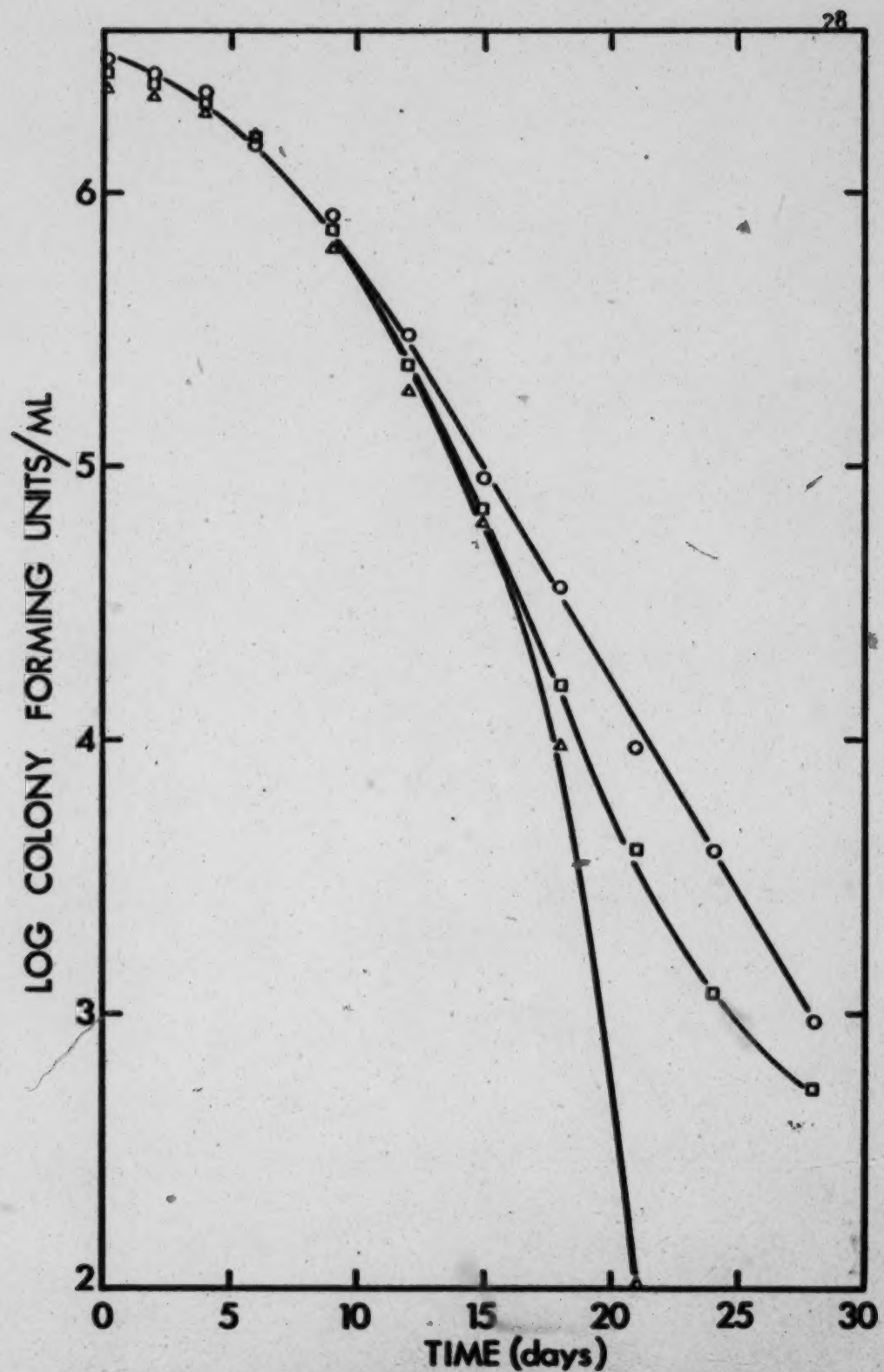

Fig. 9. Effect of storage at 4°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 7.2). Plating media ○—TSA; □—TPEY; △—MSA.

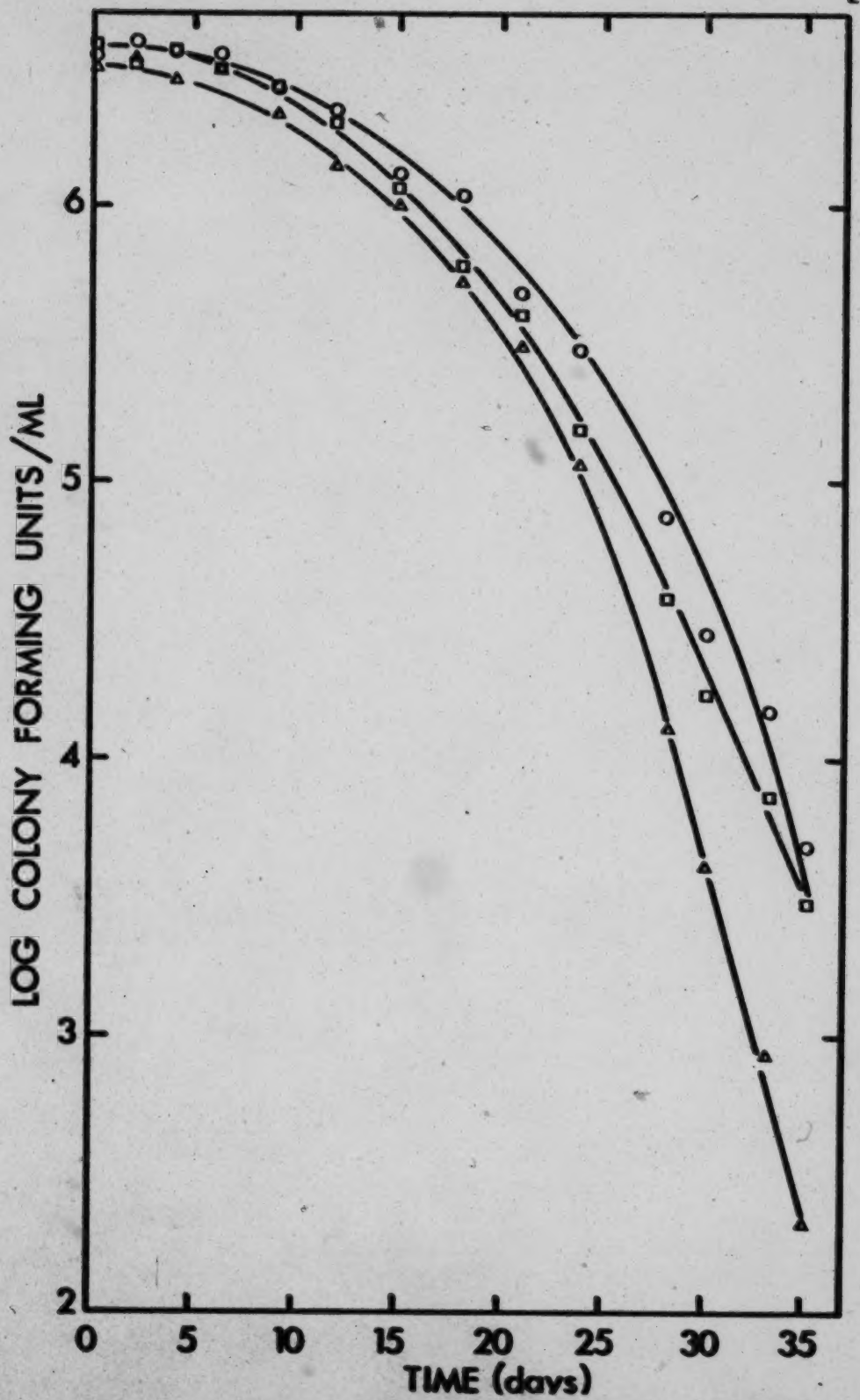

Fig. 10. Effect of storage at 4°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 5.0). Plating media o—TSA; □—TPEY; Δ—MSA.

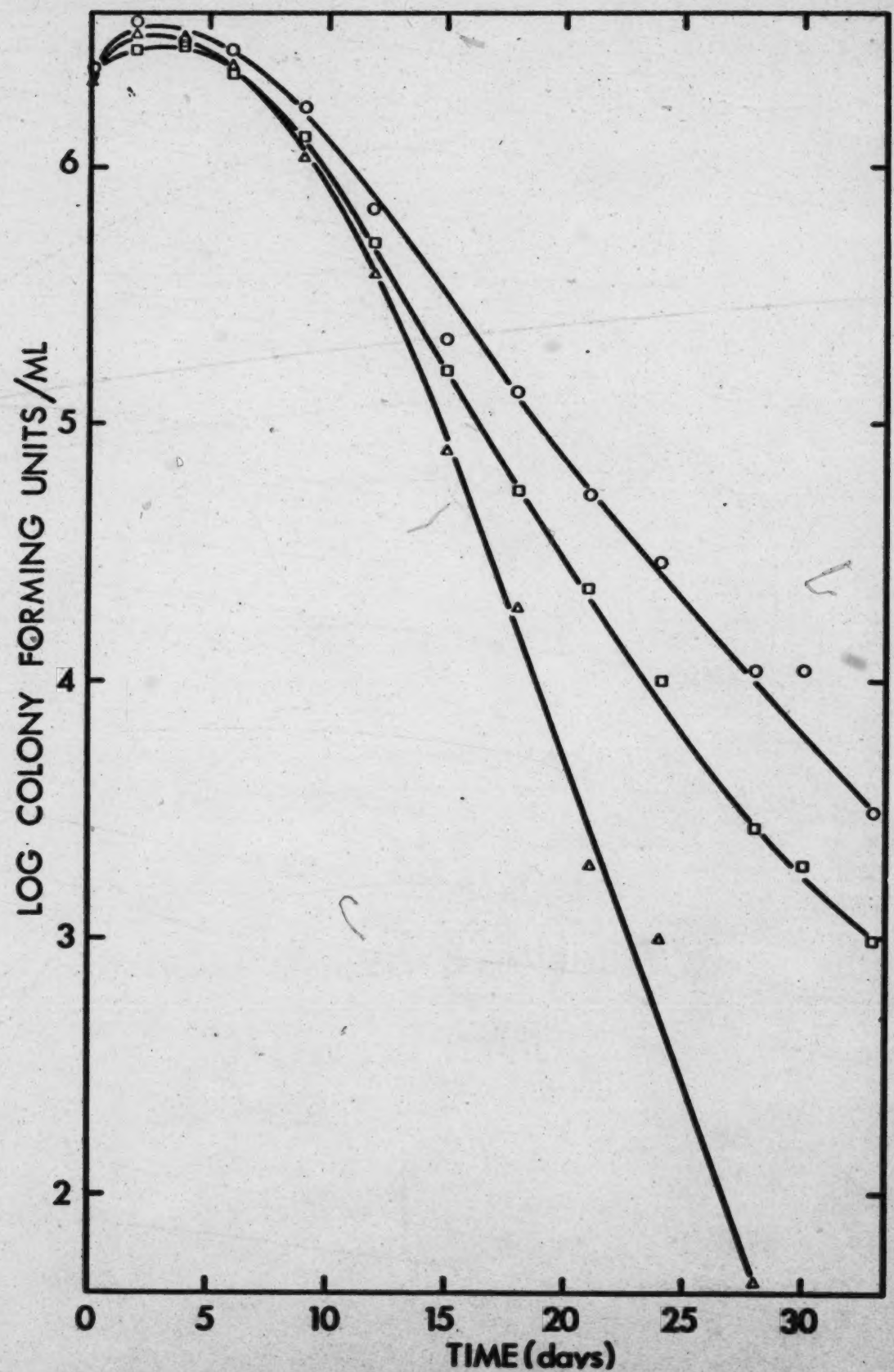

Fig. 11. Effect of storage at 4°C on the viability of a stationary phase inoculum of S. aureus in TSB (3% NaCl, pH 7.2). Plating media ○—TSA; □—TPEY; △—MSA.

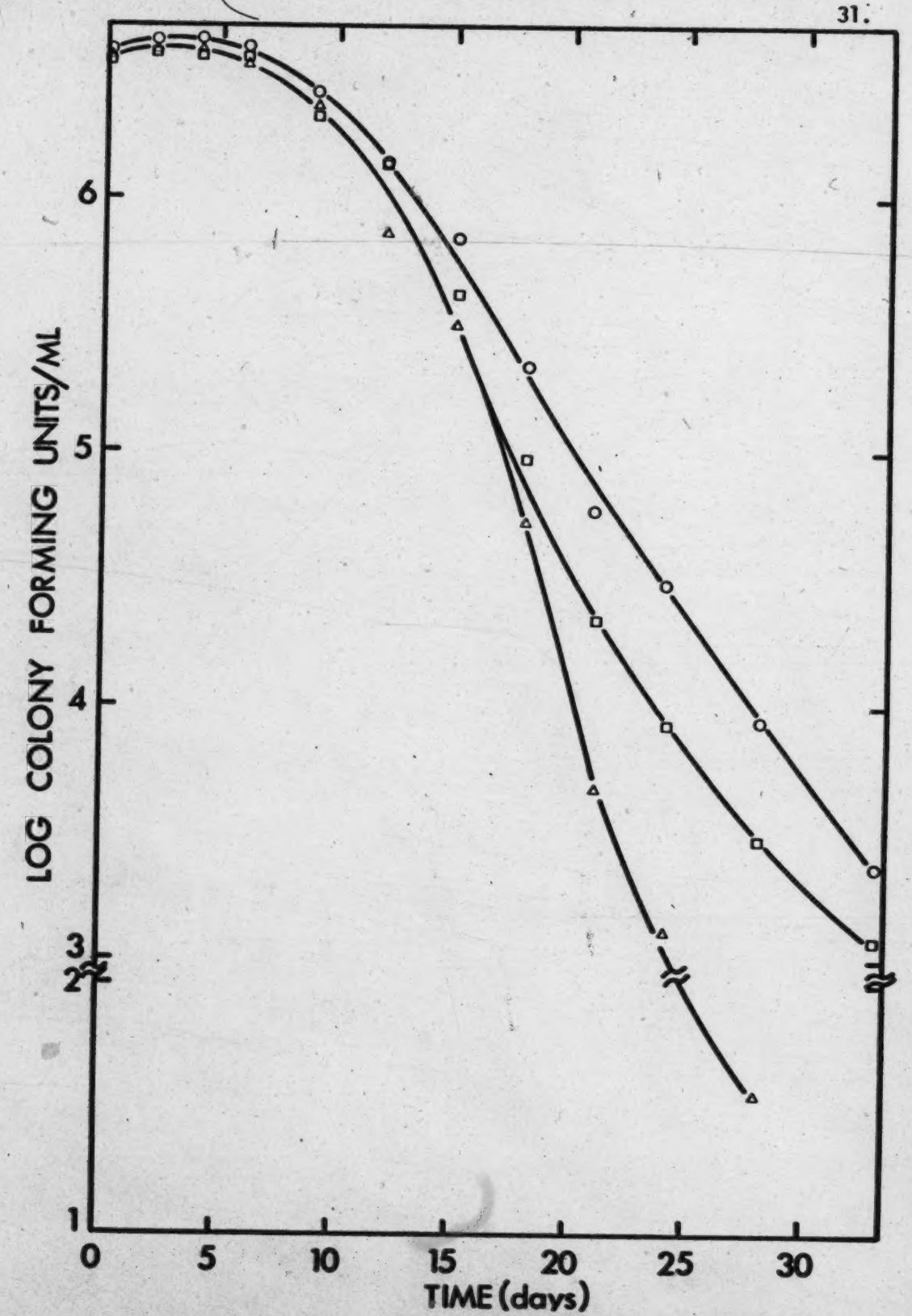

Fig. 12. Effect of storage at 4°C on the viability of a stationary phase inoculum of S. aureus in TSB (5% NaCl, pH 7.2). Plating media ○—TSA; □—TPEY; △—MSA.

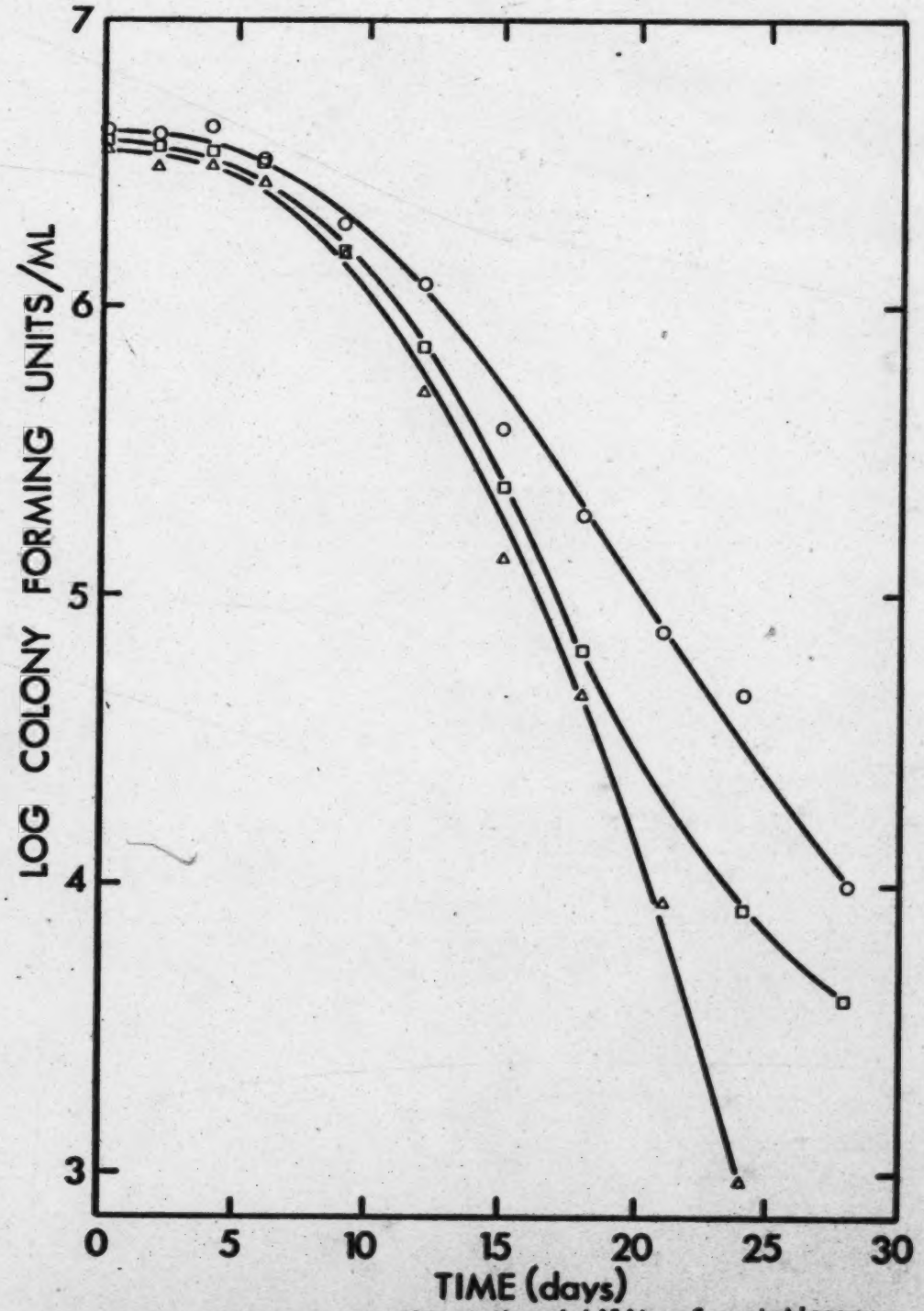

Fig. 13. Effect of storage at 4°C on the viability of a stationary phase inoculum of S. aureus in TSB (10% NaCl, pH 7.2). Plating media ○—TSA; □—TPEY; △—MSA.

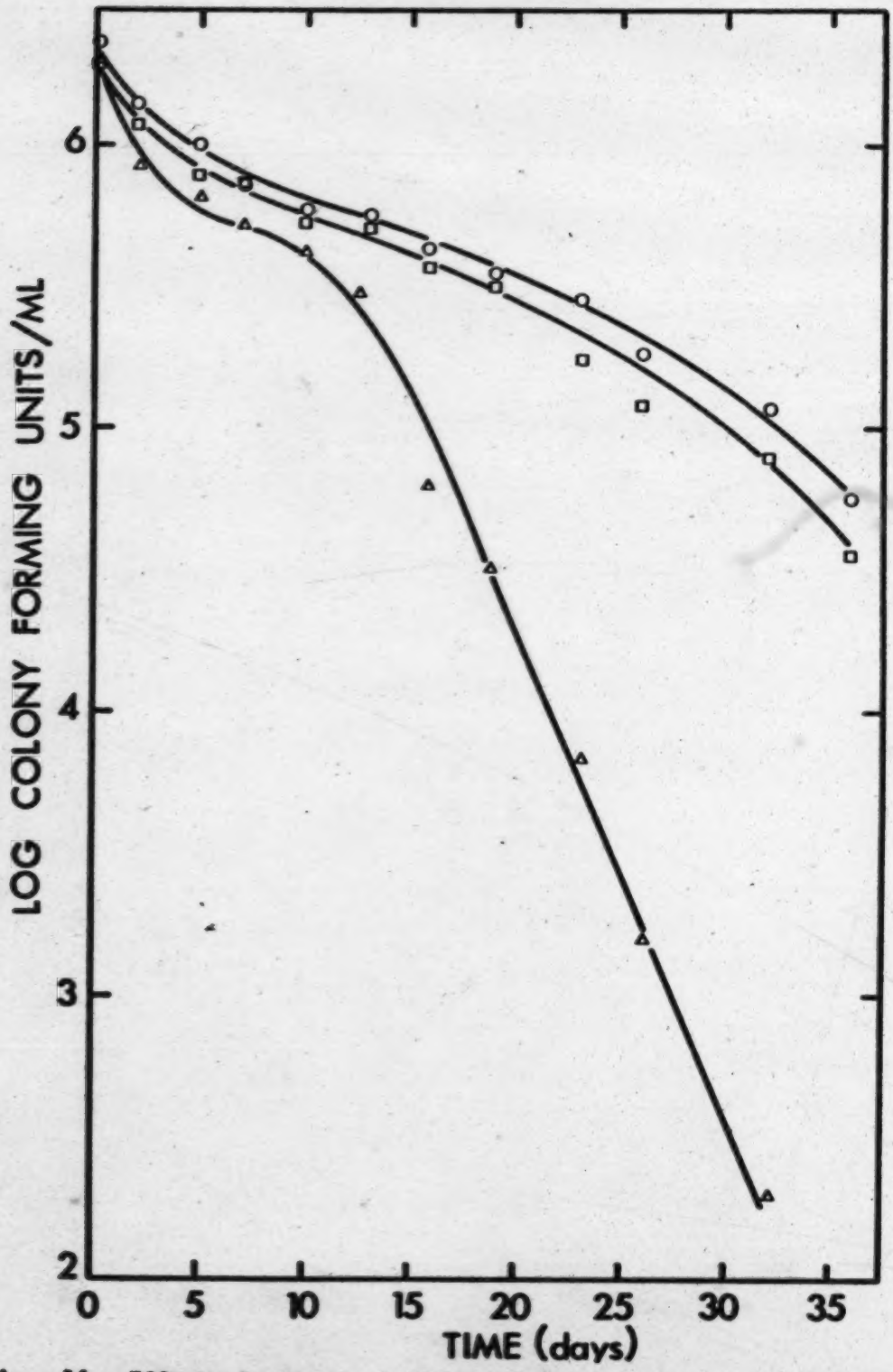

Fig. 14. Effect of storage at 1°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 7.2). Plating media ○-TSA; □-TPEY; △-MSA.

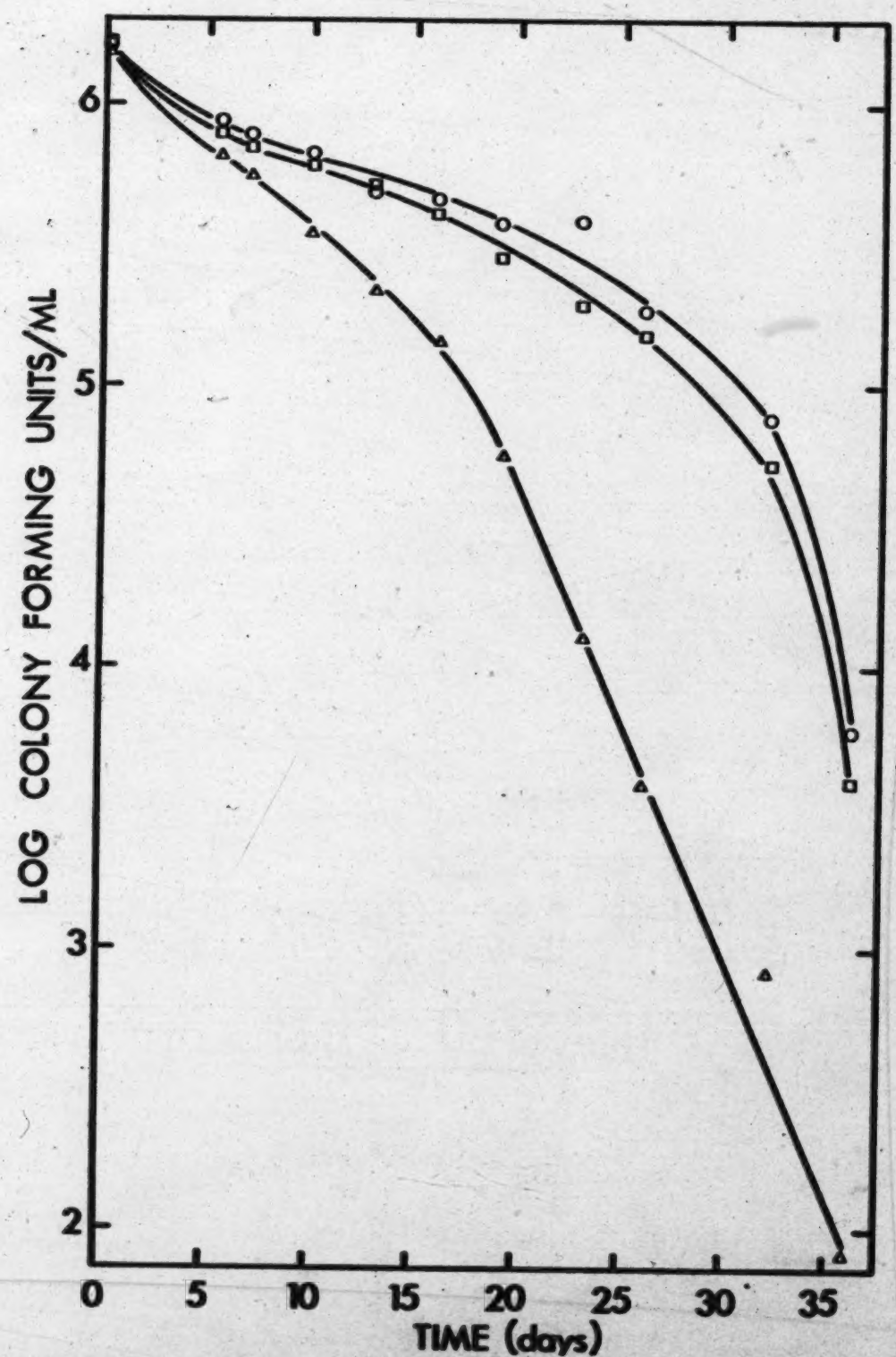

Fig. 15. Effect of storage at 3°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 7.2). Plating media ○—TSA; □—TPEY; △—MSA.

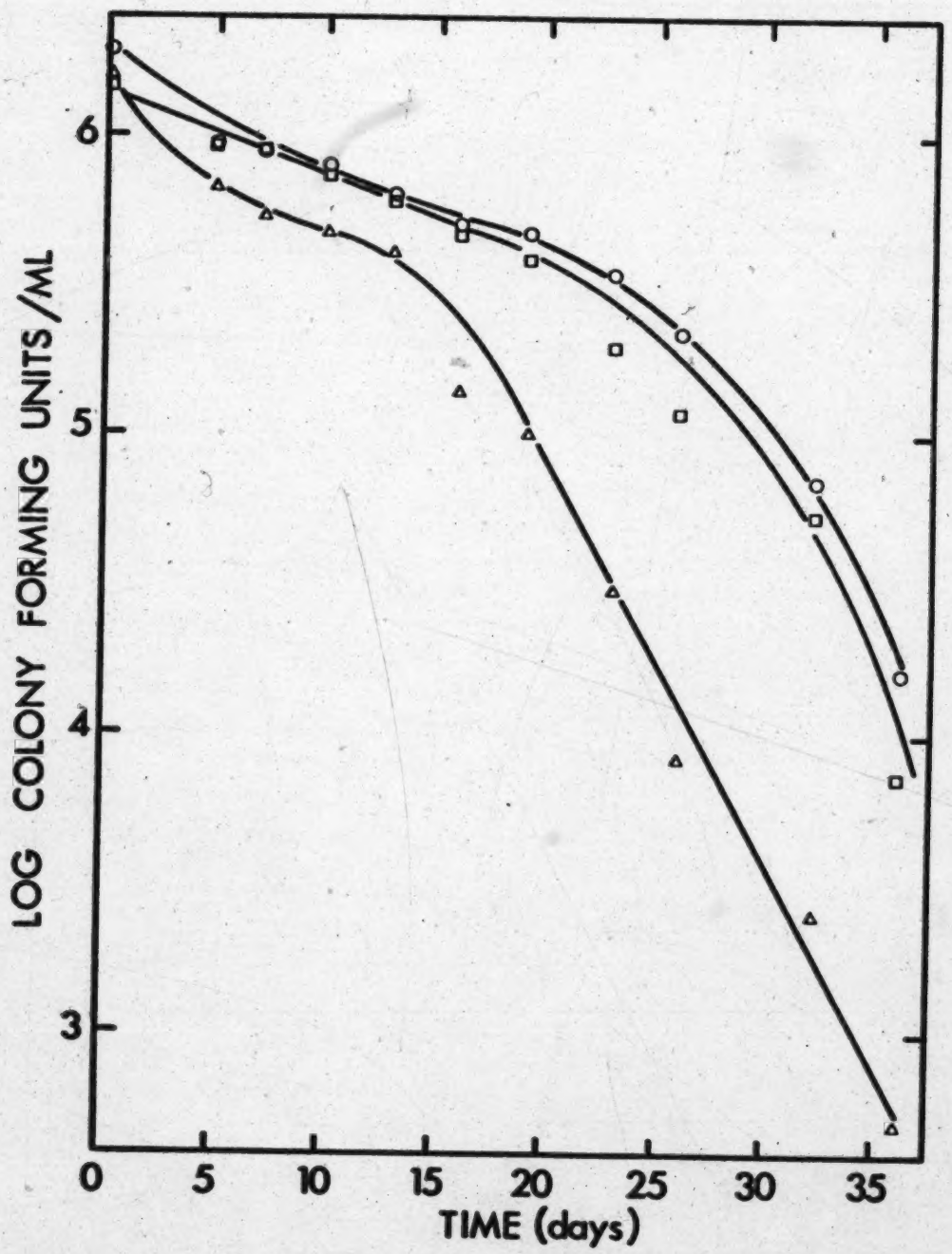

Fig. 16. Effect of storage at 5°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 7.2). Plating media ○—TSA; □—TPEY; △—MSA.

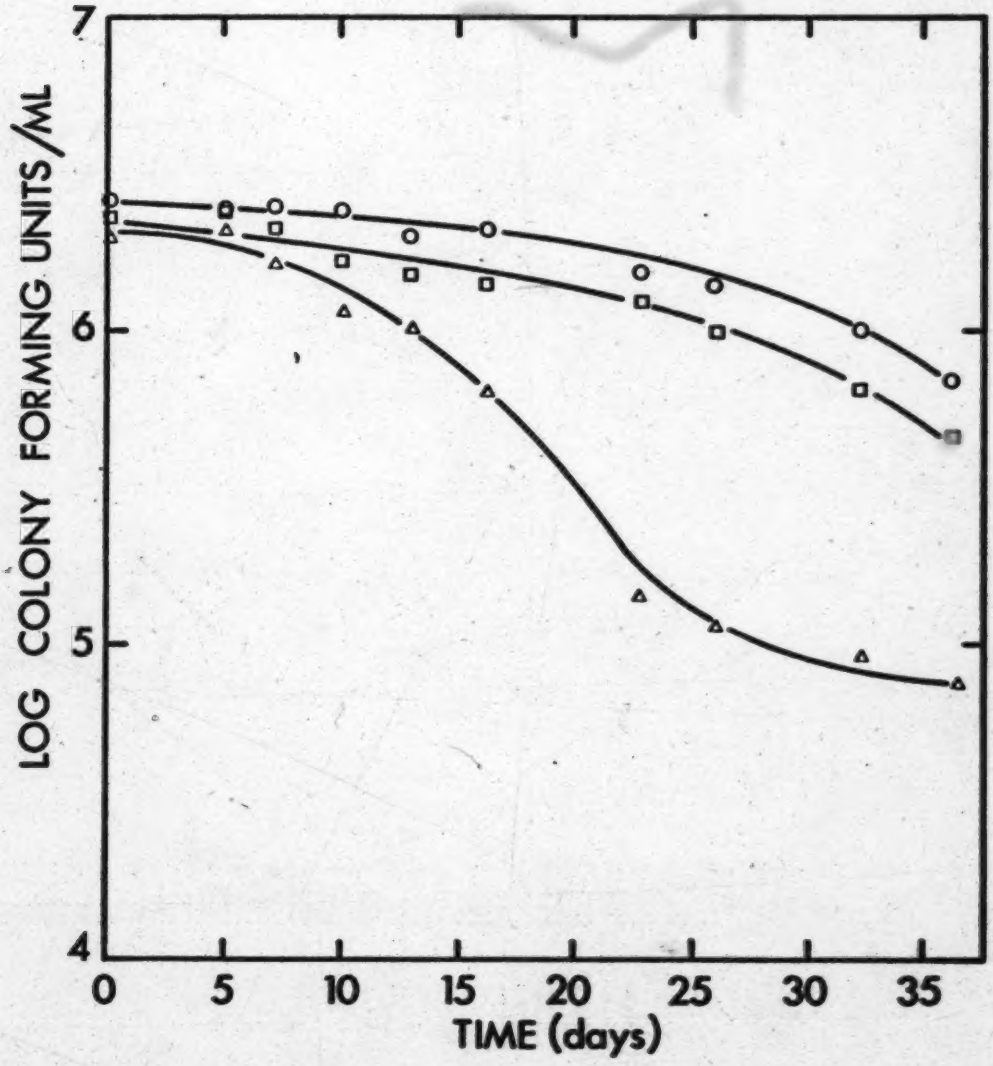

Fig. 17. Effect of storage at 7°C on the viability of a stationary phase inoculum of S. aureus in TSB (pH 7.2). Plating media ○ –TSA; □ –TPEY; △ –MSA.

(difference in counts on TSA and MSA) increases with decreasing temperature from 7°C - 1°C.

Injury to Exponential Cultures

The effect of sub-minimal temperatures was investigated on all three organisms using inocula from the exponential phase of growth. 1000 ml quantities of TSB (pH 7.2 or 5.0) in 2 l Erlenmeyer flasks in the plexiglass baths were inoculated at the 1% level.. S. aureus and E. coli were incubated at 4°C; Str. faecalis at 1°C. S. aureus in the exponential phase showed injury after 2 days storage at pH 7.2 and after 12 days a difference of >3 log cycles occurred between counts on TSA and MSA, but no injury was demonstrated on TPEY (Fig. 18). A comparison of Fig. 18 with Figs. 1 and 9 shows that S. aureus in the exponential phase of growth loses its ability to grow on MSA much more rapidly and to a greater extent than stationary phase cultures. The organism tends to die, i.e., lose its ability to grow on TSA more rapidly if it is exposed to low temperatures at the stationary phase of growth. At pH 5.0, S. aureus in the exponential phase is also much more sensitive to injury than in the stationary phase as seen from the count on TSA v MSA, but no injury was apparent with TPEY (Fig. 19).

E. coli in the exponential phase died very rapidly at both pH 7.2 and 5.0 when stored at 4°C and a difference of 1 log cycle or greater between counts on TSA and VRBA was demonstrated at 2 days storage (Figs. 20 and 21). Counts on both TSA and VRBA decreased more rapidly at pH 7.2 than at pH 5.0.

It may be seen from Figs. 22 and 23 that no demonstratable injury occurred to Str. faecalis in the exponential phase of growth

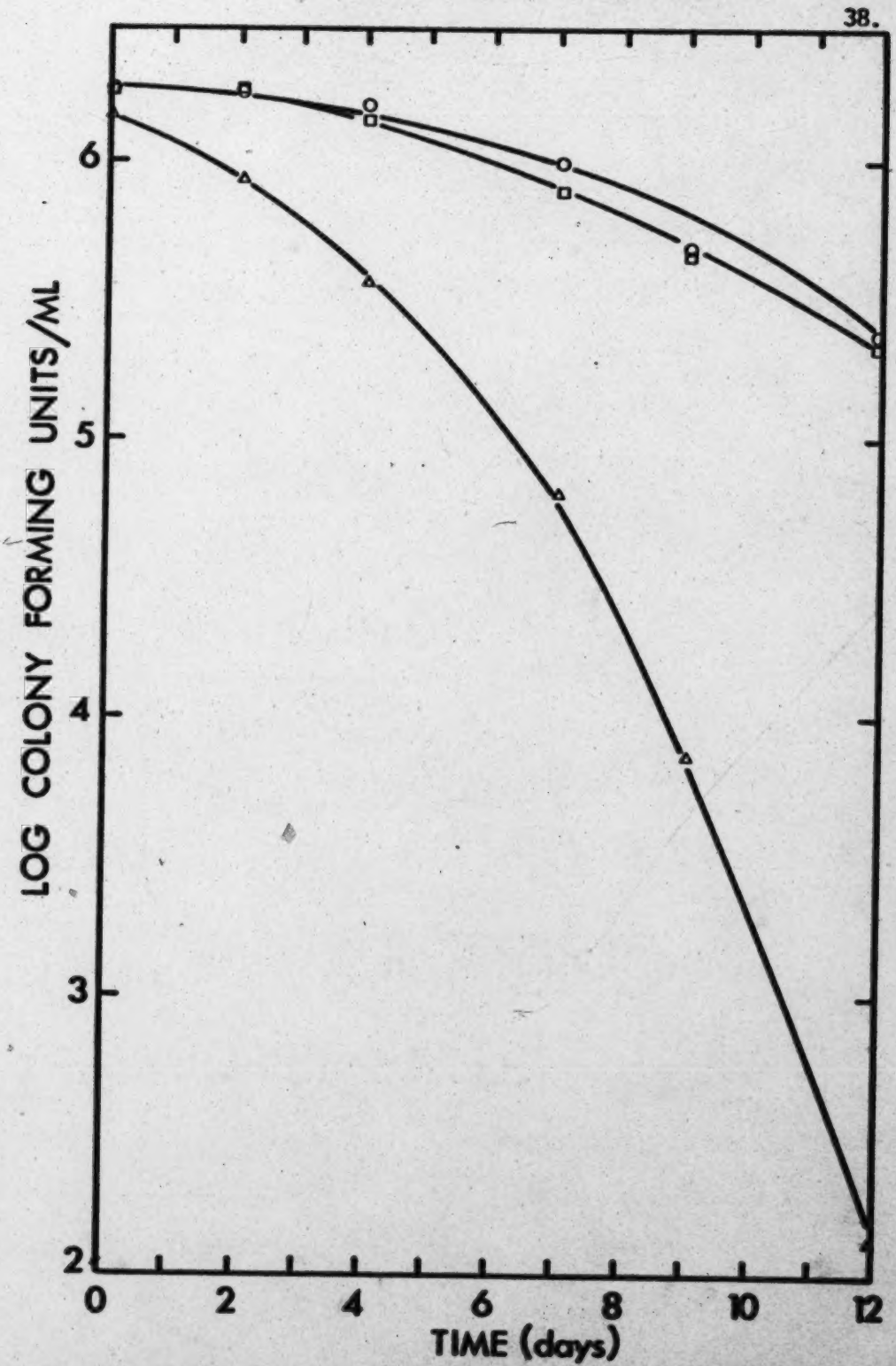

Fig. 18. Effect of storage at 4°C on the viability of an exponential phase inoculum of S. aureus in TSB (pH 7.2). Plating media o—TSA; □—TPEY; △—MSA.

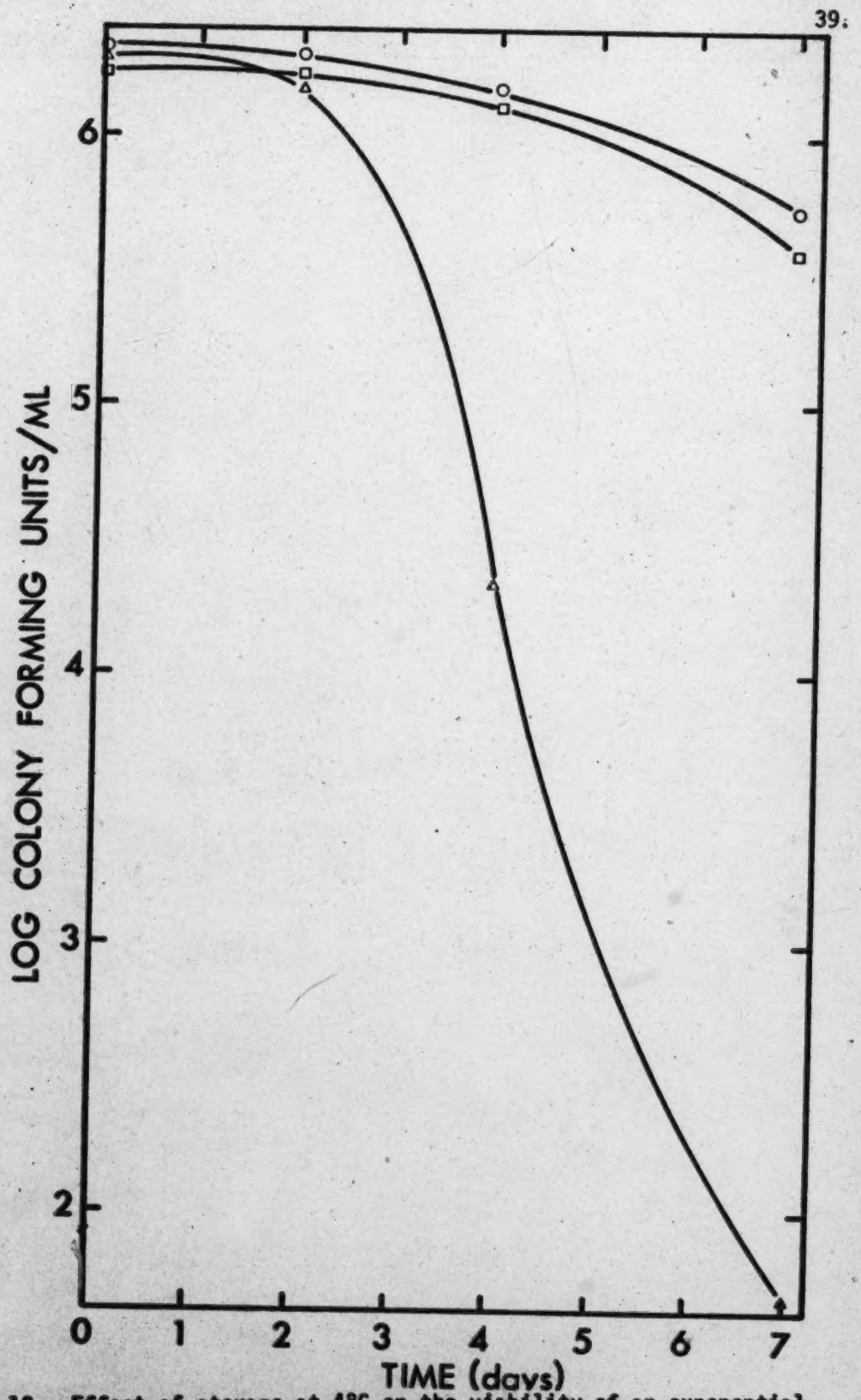

Fig. 19. Effect of storage at 4°C on the viability of an exponential phase inoculum of S. aureus in TSB (pH 5.0). Plating media ○—TSA; □—TPEY; △—MSA.

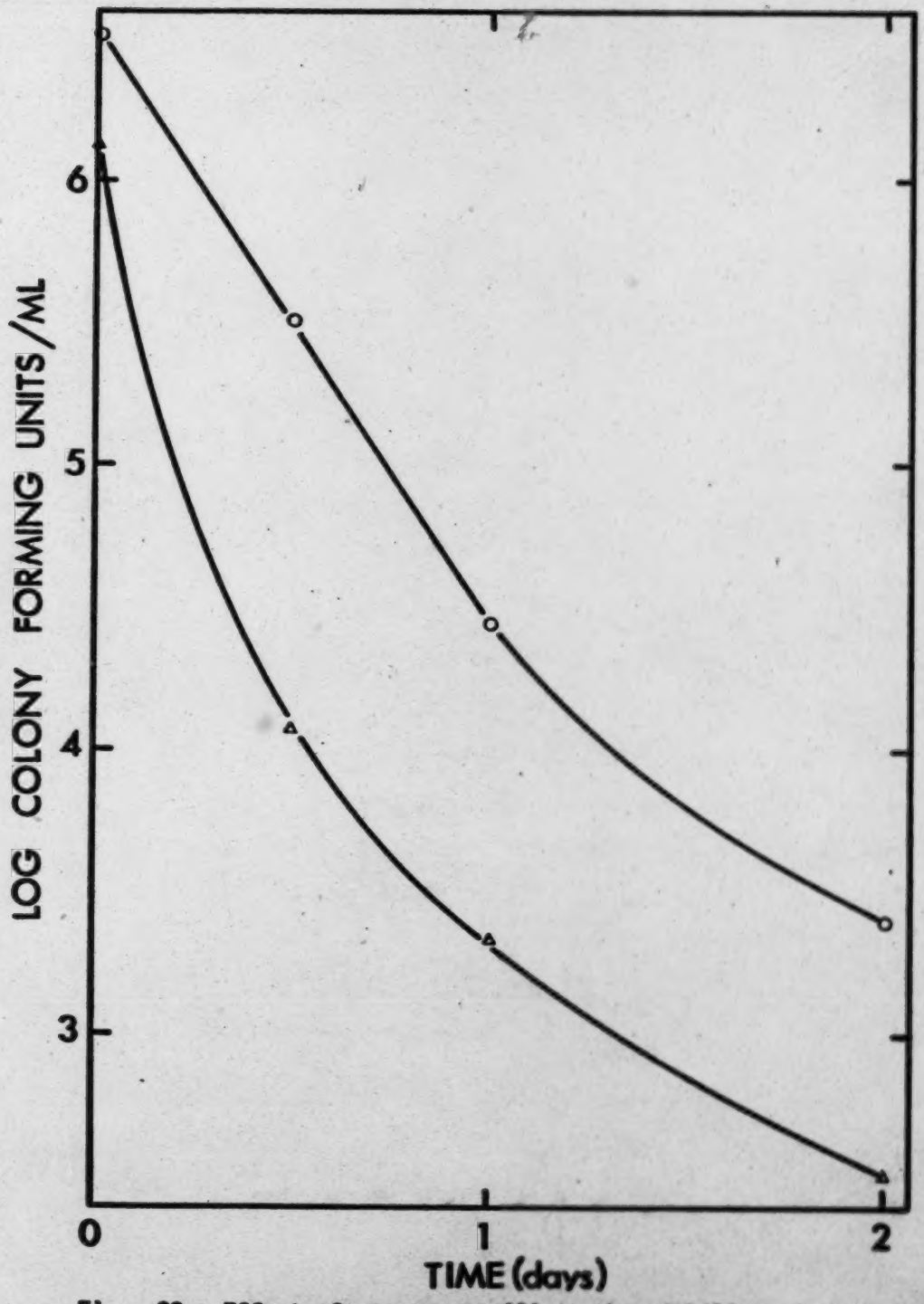

Fig. 20. Effect of storage at 4°C on the viability of an exponential phase inoculum of E. coli in TSB (pH 7.2). Plating media ○ – TSA; △ – VRBA.

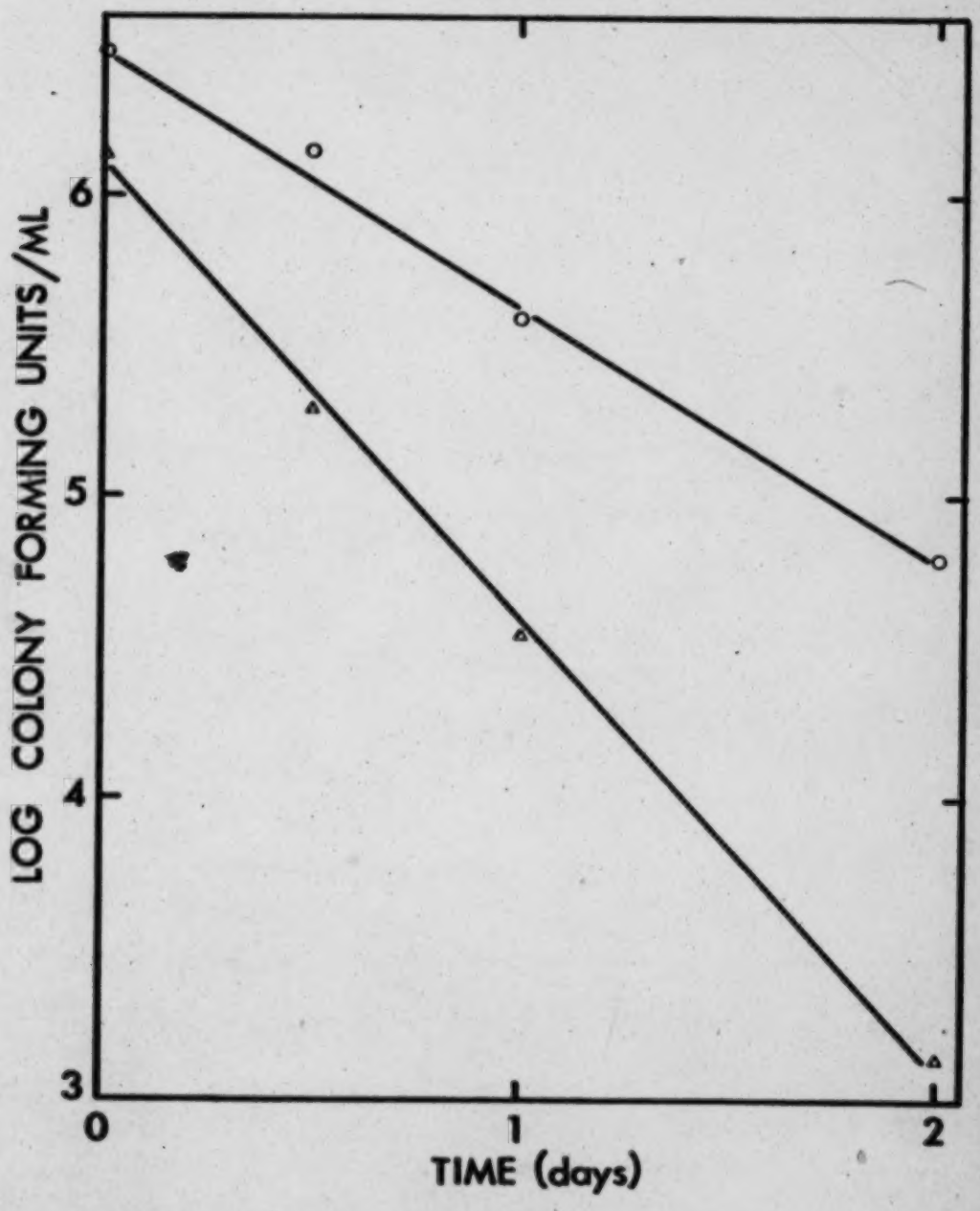

Fig. 21. Effect of storage at 4°C on the viability of an exponential phase inoculum of E. coli in TSB (pH 5.0). Plating media o — TSA; Δ — VRBA.

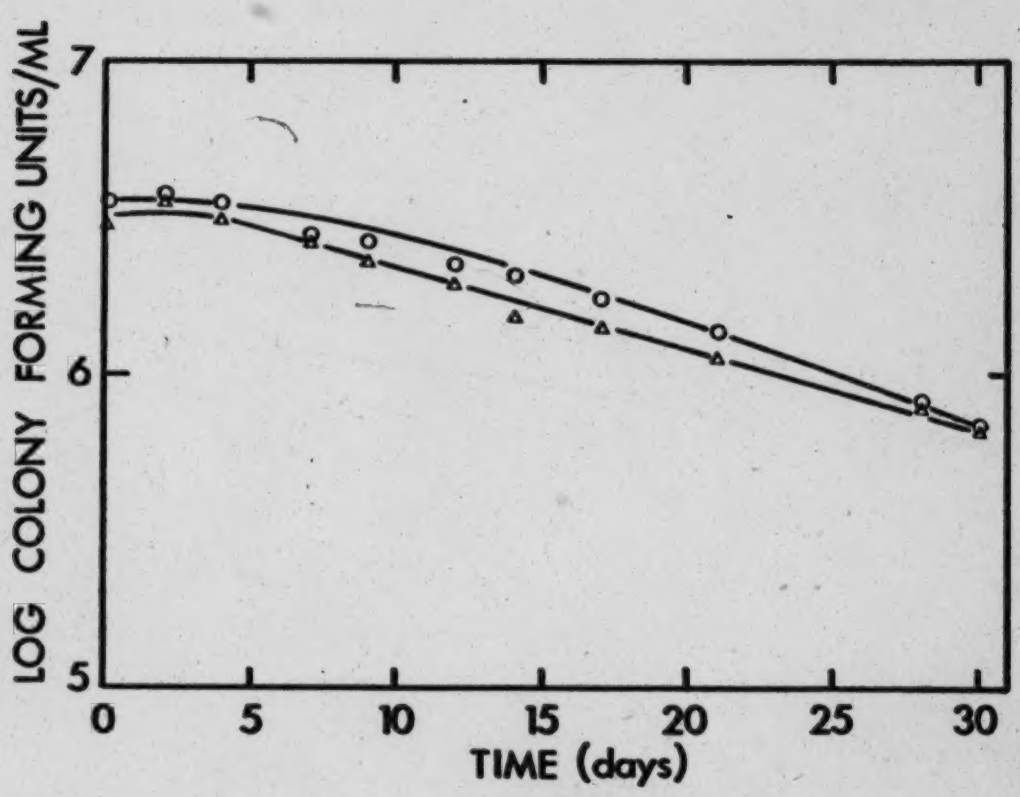

Fig. 22. Effect of storage at 1°C on the viability of an exponential phase inoculum of Str. faecalis in TSB (pH 7.2). Plating media o — TSA; Δ — KFA.

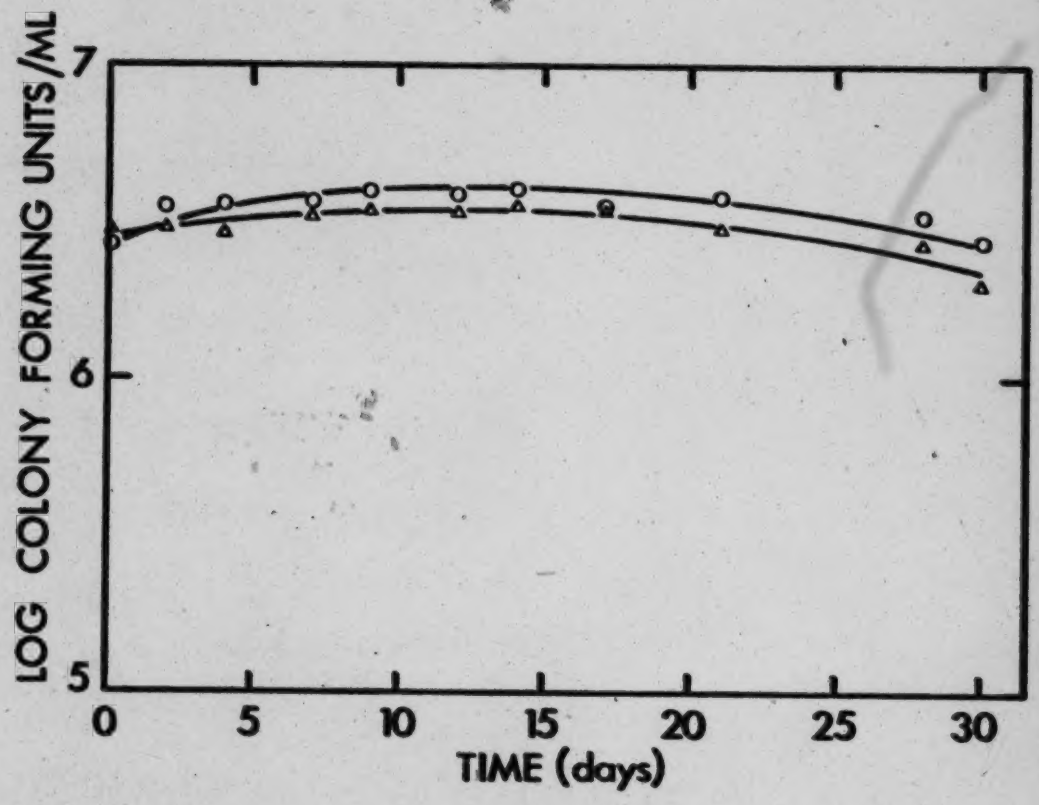

Fig. 23. Effect of storage at 1°C on the viability of an exponential phase inoculum of Str. faecalis in TSB (pH 5.0). Plating media o—TSA; Δ—KFA.

even after storage for 30 days at 1°C.

Temperature Effects on Growth and Injury using a Temperature Gradient Incubator

Tryptic Soy Broth (pH 7.2) in Astell roll tubes was inoculated at the 1% level with exponential cultures of S. aureus and E. coli and incubated in a temperature gradient incubator. The viable count was determined on selective and non-selective media at the start of the experiment and subsequently at regular intervals.

The final counts are illustrated in Figs. 24 and 25. After 12 days incubation, injury to S. aureus was demonstrated at 0, 1.0, 2.5, 3.5, 5.5 and 6.5°C (Fig. 24). No growth or injury occurred at 8.0°C but an increase in cell numbers was apparent at 9.0 and 10.5°C. From these results it would appear that the minimum growth temperature for the organism under these conditions lies between 8.0 and 9.0°C while the temperature required to initiate injury lies between 6.5 and 8.0°C.

After 5 days incubation E. coli exhibited no growth or injury at 5.5°C while growth was demonstrated at 6.5°C and higher and injury was apparent at 3.5°C and lower (Fig. 25).

Recovery from Injury

Growth of Uninjured Cells

Growth curves illustrated in Figs. 26 - 31 show that uninjured cells of S. aureus and E. coli grow equally well on the complex medium as on the selective media, under the conditions of temperature, pH and inoculation used to assess the ability of injured cells to recover their tolerance to the selective media. They also serve as controls in

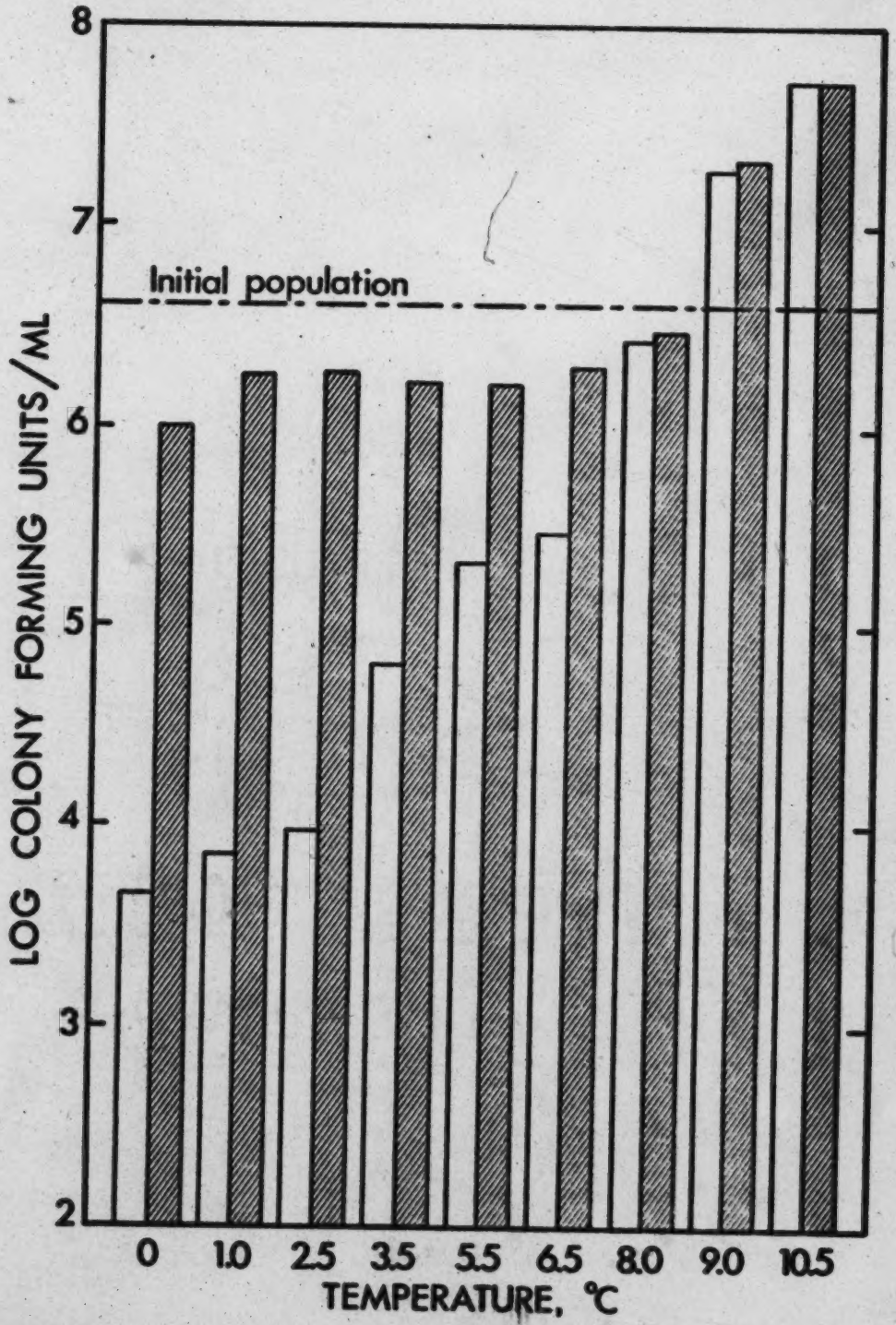

Fig. 24. Effect of temperature on growth and viability of S. aureus in TSB (pH 7.2). Incubation period 12 days. Plating media, shaded - TSA; open - MSA.

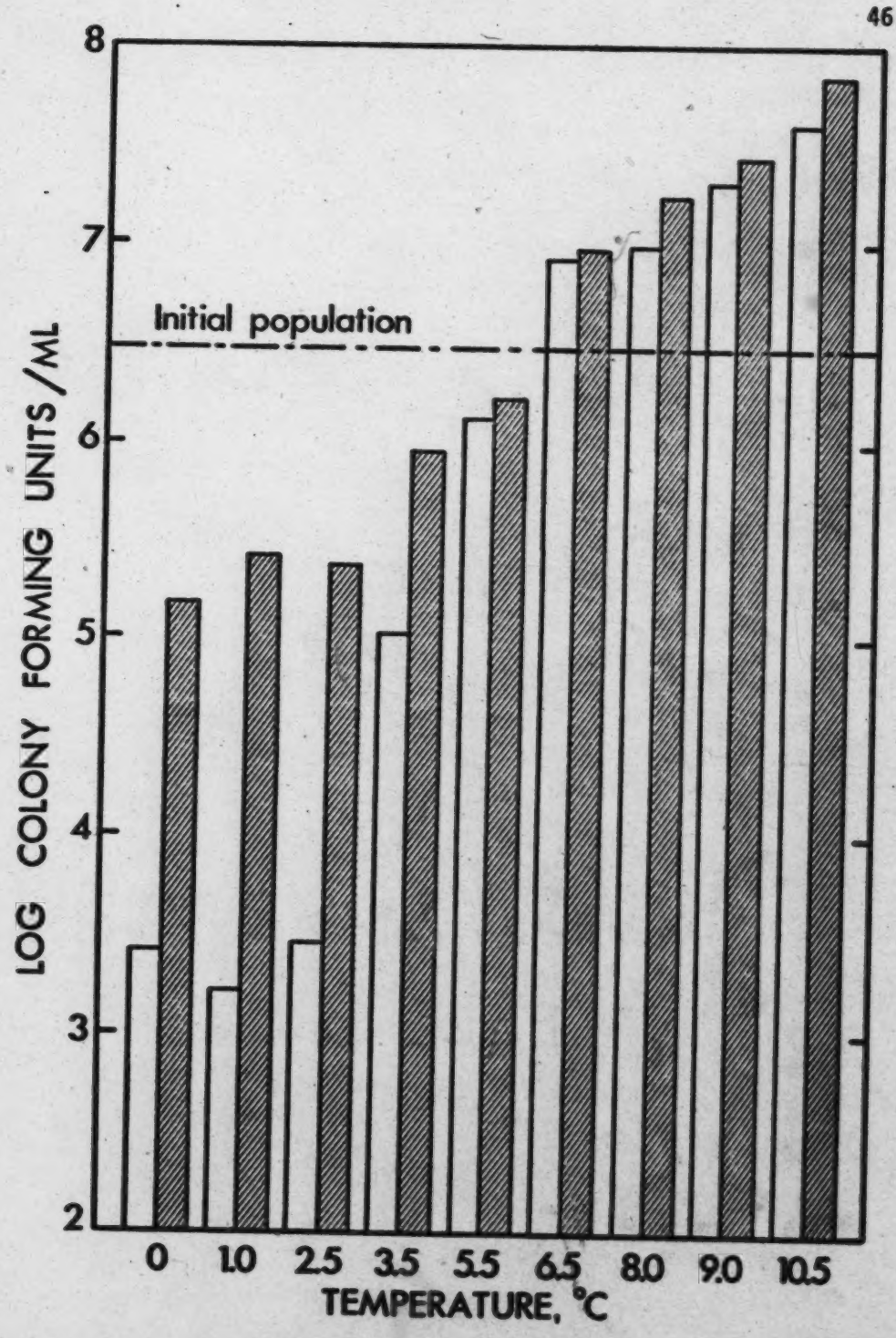

Fig. 25. Effect of temperature on growth and viability of E. coli in TSB (pH 7.2). Incubation period 5 days. Plating media, shaded - TSA; open - VRBA.

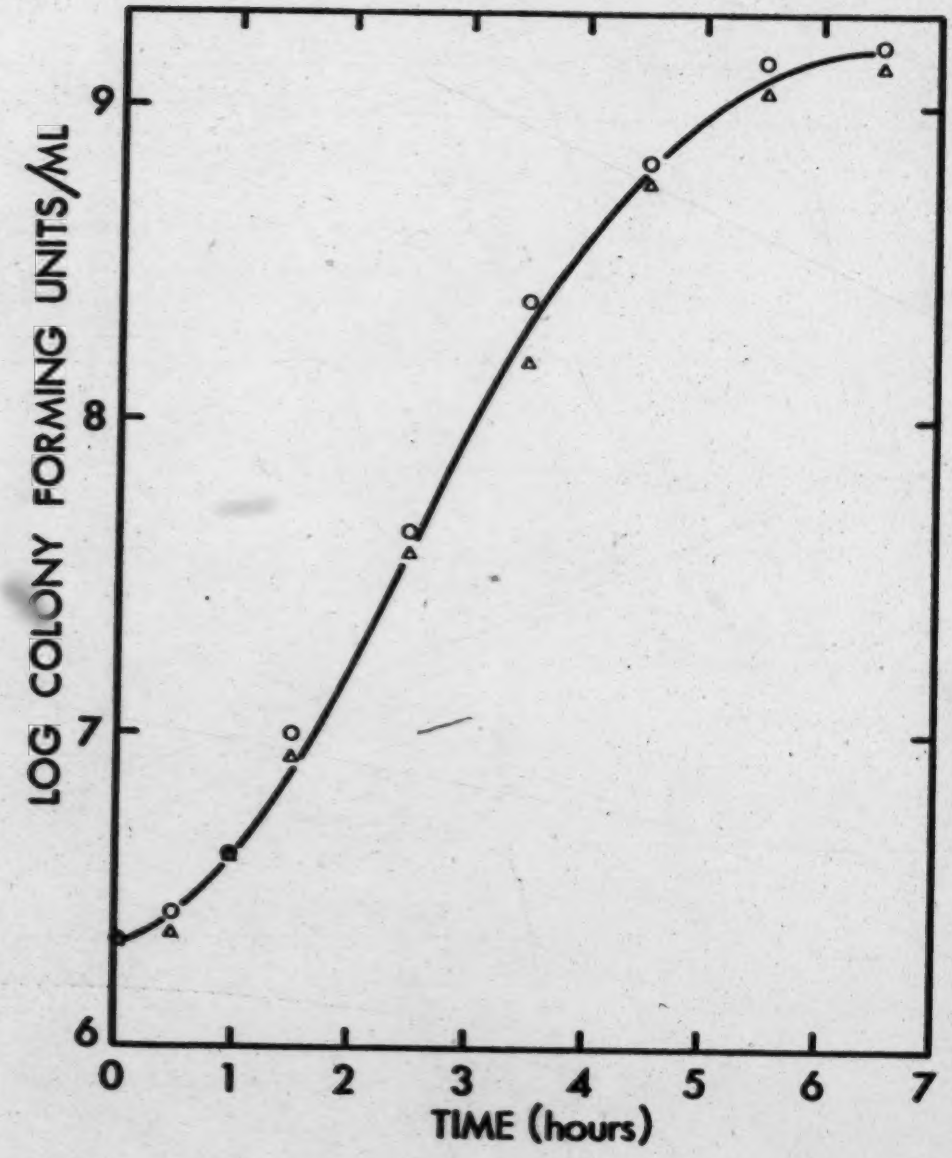

Fig. 26. Growth of a stationary phase inoculum (0.1%) of S. aureus in TSB (pH 7.2) at 37°C. Plating media, o — TSA; Δ — MSA.

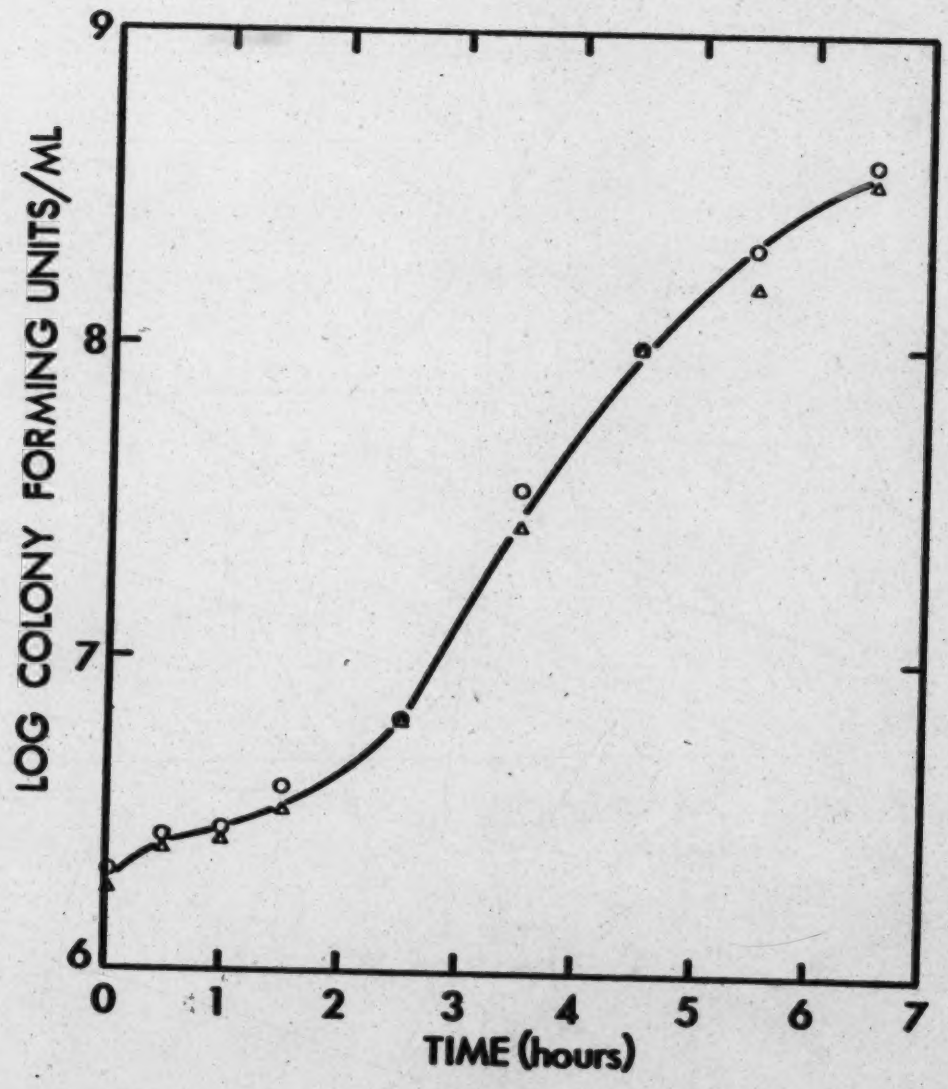

Fig. 27. Growth of a stationary phase inoculum (0.1%) of S. aureus in TSB (pH 5.0) at 37°C. Plating media, o — TSA; Δ — MSA.

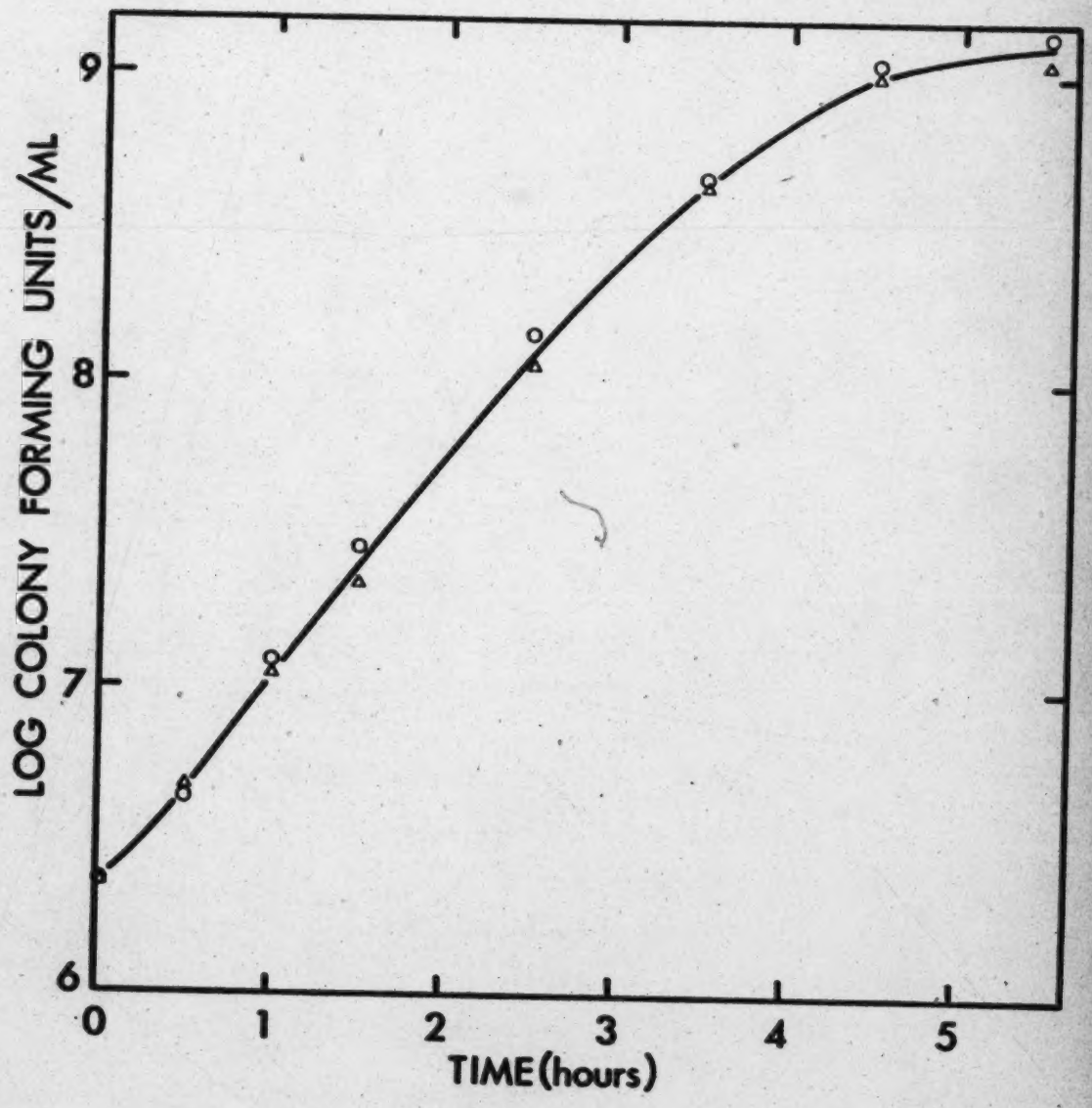

Fig. 28. Growth of an exponential phase inoculum (1%) of S. aureus in TSB (pH 7.2) at 37°C. Plating media, o — TSA; Δ — MSA.

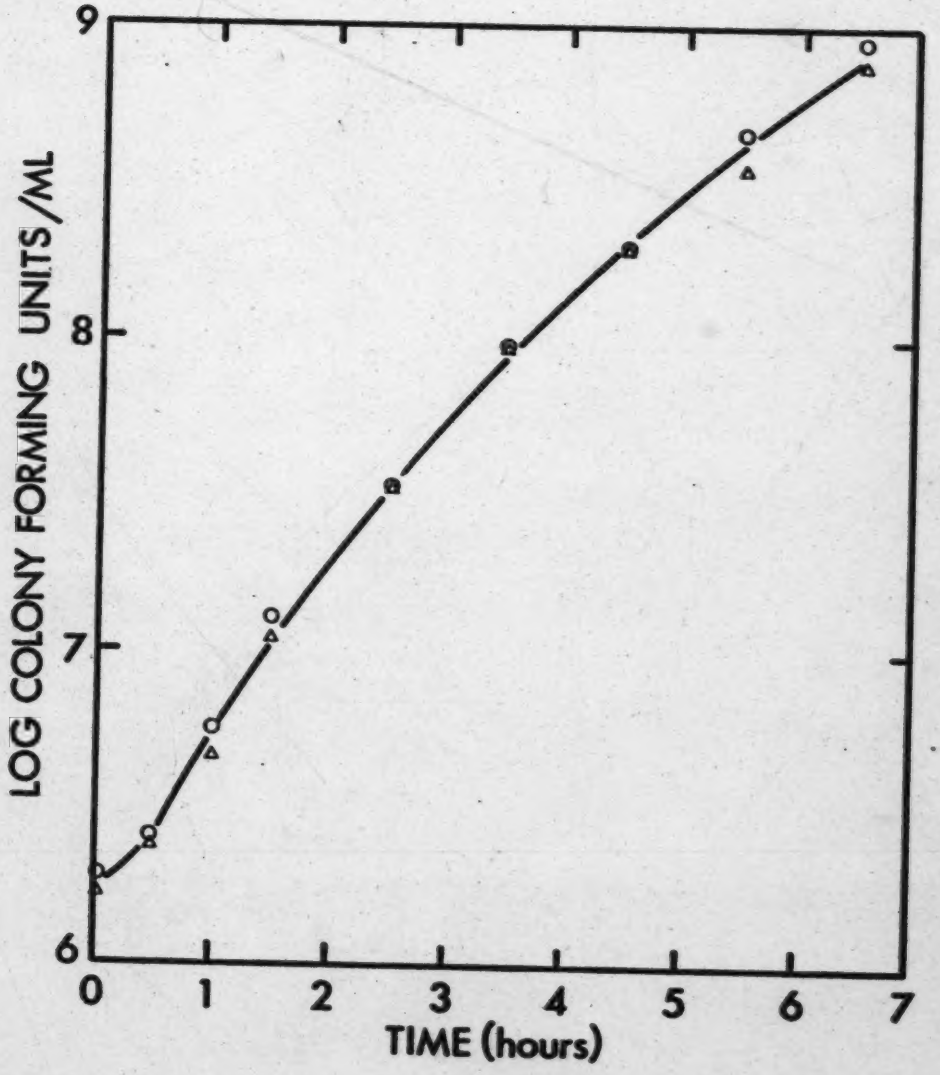

Fig. 29. Growth of an exponential phase inoculum (1%) of S. aureus in TSB (pH 5.0) at 37°C. Plating media, o — TSA; Δ — MSA.

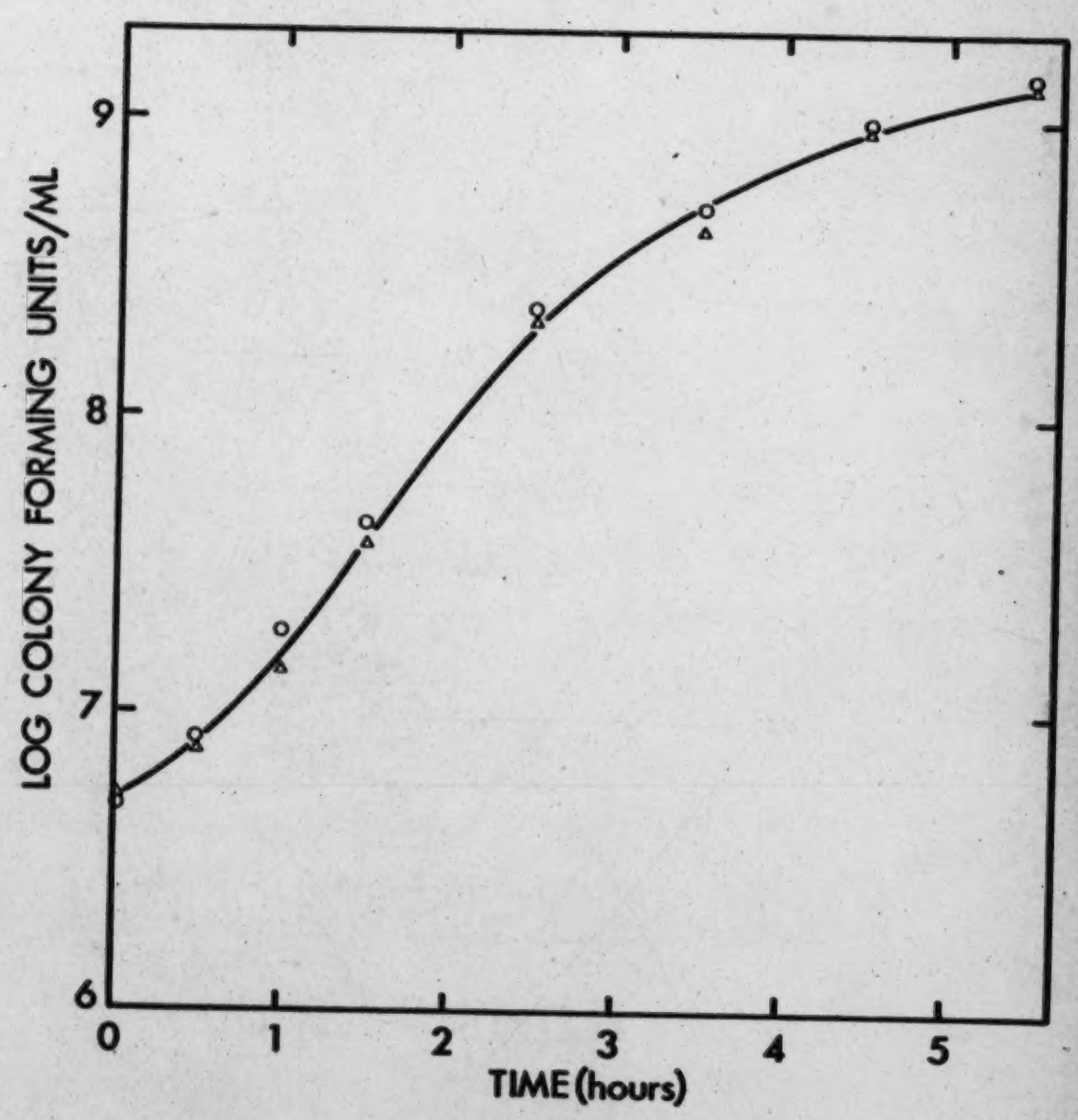

Fig. 30. Growth of an exponential phase inoculum (1%) of E. coli in TSB (pH 7.2) at 37°C. Plating media, o—TSA; Δ— VRBA.

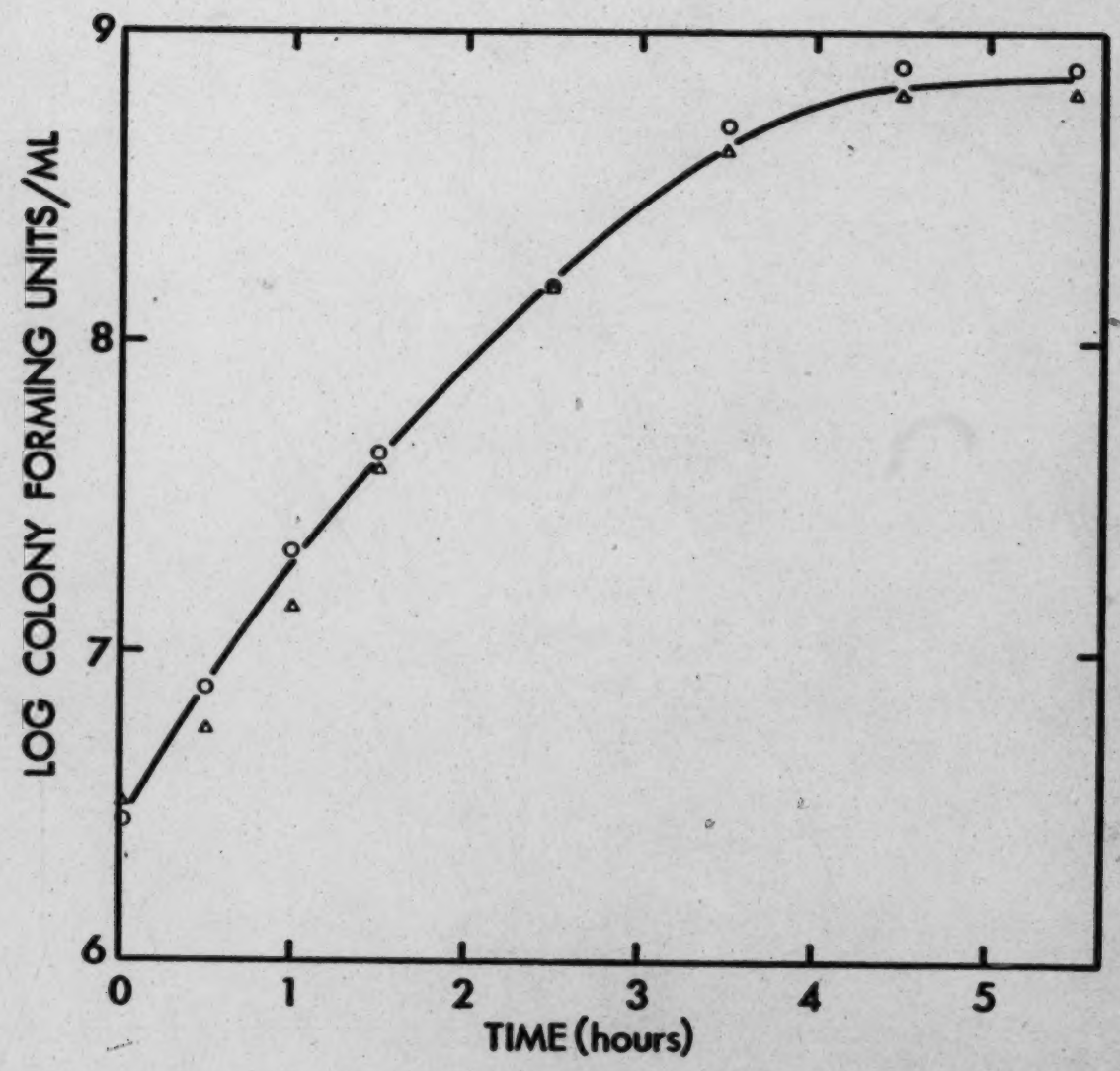

Fig. 31. Growth of an exponential phase inoculum (1%) of E. coli in TSB (pH 5.0) at 37°C. Plating media, o — TSA; Δ — VRBA.

evaluating the cultural responses of the organisms after storage at sub-minimal temperatures.

Recovery of S. aureus

The recovery of an 18 h inoculum of S. aureus injured by storage in TSB (pH 7.2) at 4°C for 24 days is illustrated in Fig. 32. The recovery temperature was 37°C. The lag phase of the organism is markedly extended (Fig. 26 v Fig. 32). During the prolonged lag phase the organism recovered its ability to reproduce on MSA and recovery took place before growth was initiated. The relatively stable count on TSA during the lag phase indicates that no reversible modification of the organism's ability to grow on TSA occurred during storage. 70% recovery occurred during the first 1 1/2 hours incubation, but 3 - 4 h incubation was required for complete recovery.

The recovery of a similar inoculum of S. aureus injured by storage at 4°C for 24 days on TSB at a pH of 5.0 is shown in Fig. 33. At pH 5.0 injured cells of S. aureus did not recover their ability to grow on MSA when incubated at 37°C. Instead the injured cells appeared more sensitive to the low pH and died off rapidly during the lag period after which growth of the uninjured cells was initiated. This can be seen from the rapid decline in the count on TSA while the count on MSA, after an initial drop, remains relatively stable.

The ability of exponential inocula of S. aureus to recover from injury induced by 6 days storage at 4°C in TSB (pH 7.2 and 5.0) is shown in Figs. 34 and 35. At pH 7.2 approximately 70% recovery occurred within 15 minutes upon incubation at 37°C and an extended lag phase was apparent (Fig. 34).

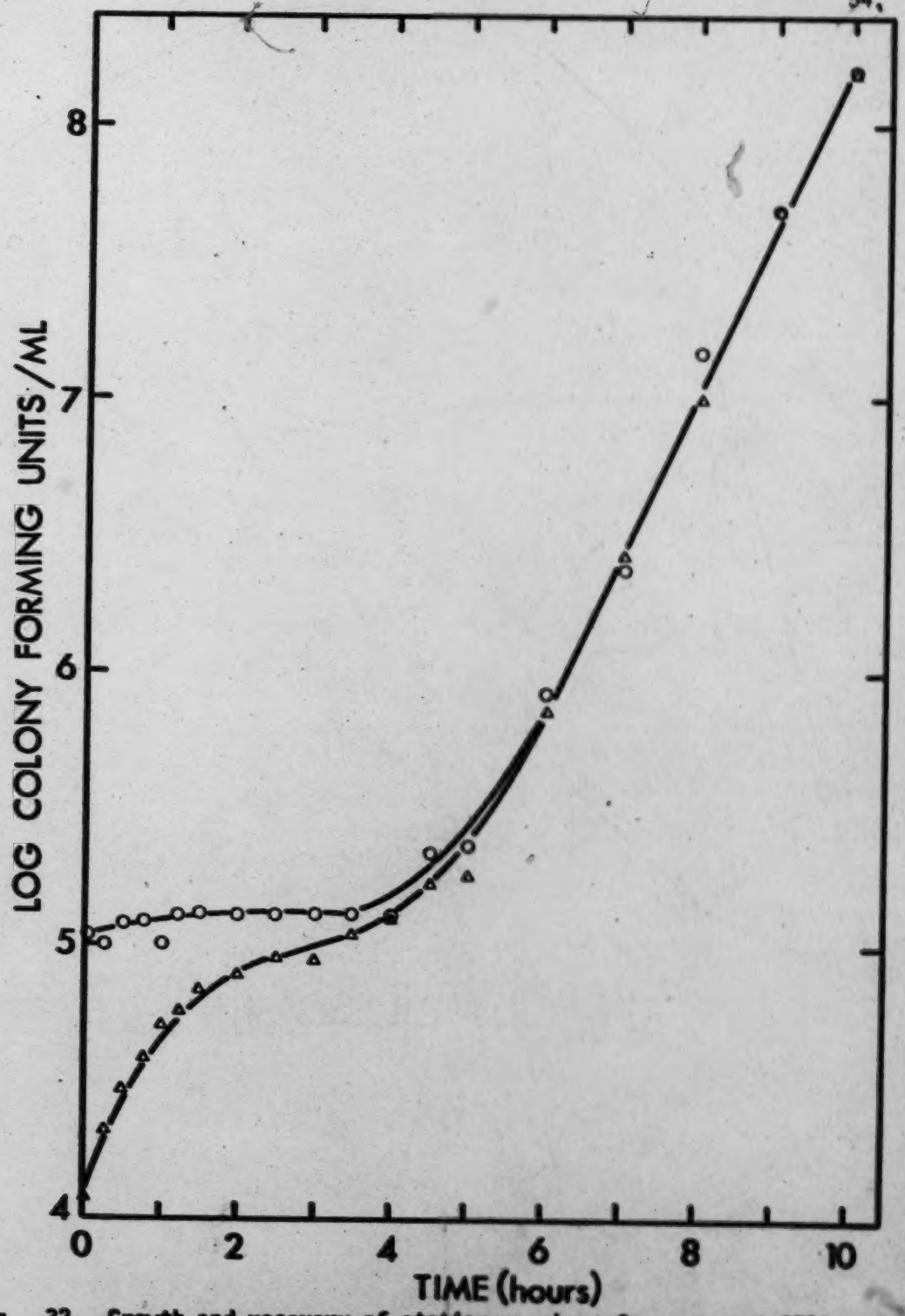

Fig. 32. Growth and recovery of stationary phase S. aureus at 37°C in TSB (pH 7.2) after exposure to 4°C for 24 days. Plating media, o — TSA; Δ — MSA.

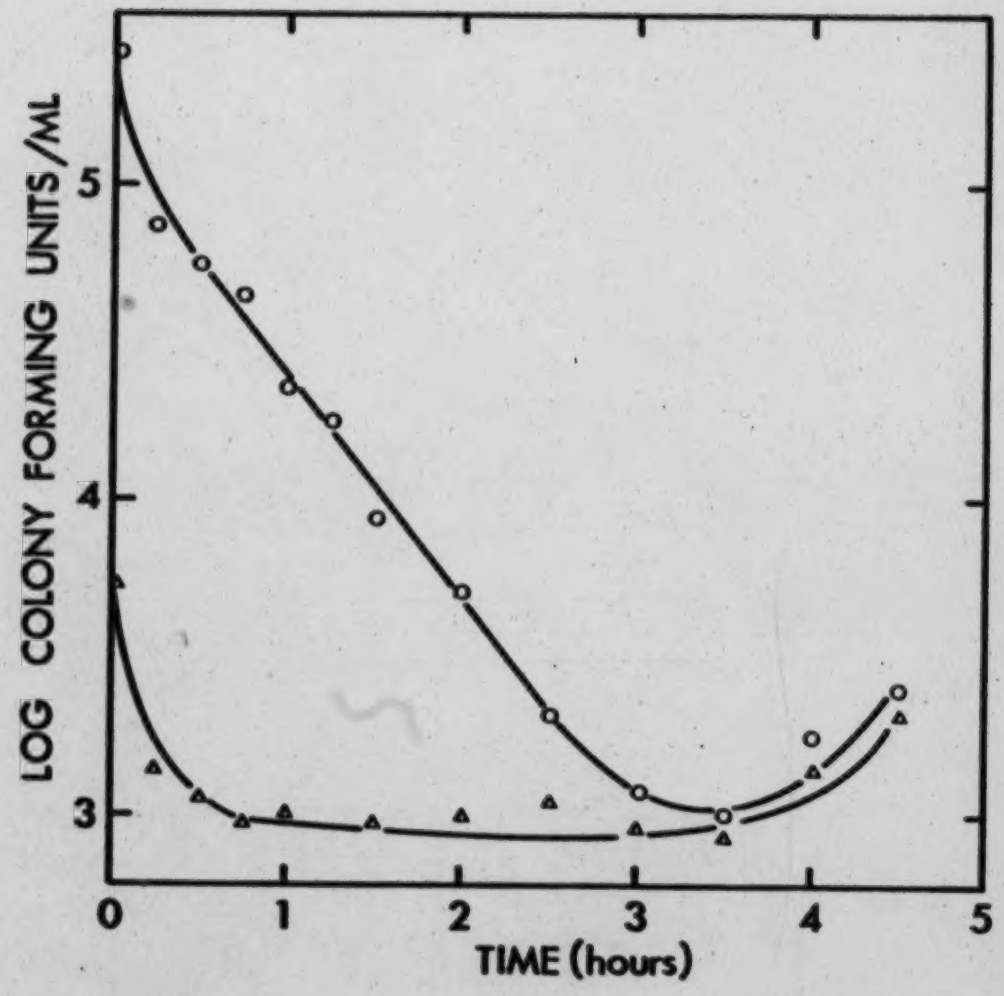

Fig. 33. Growth and recovery of stationary phase S. aureus at 37°C in TSB (pH 5.0) after exposure to 4°C for 24 days. Plating media, o — TSA; Δ — MSA.

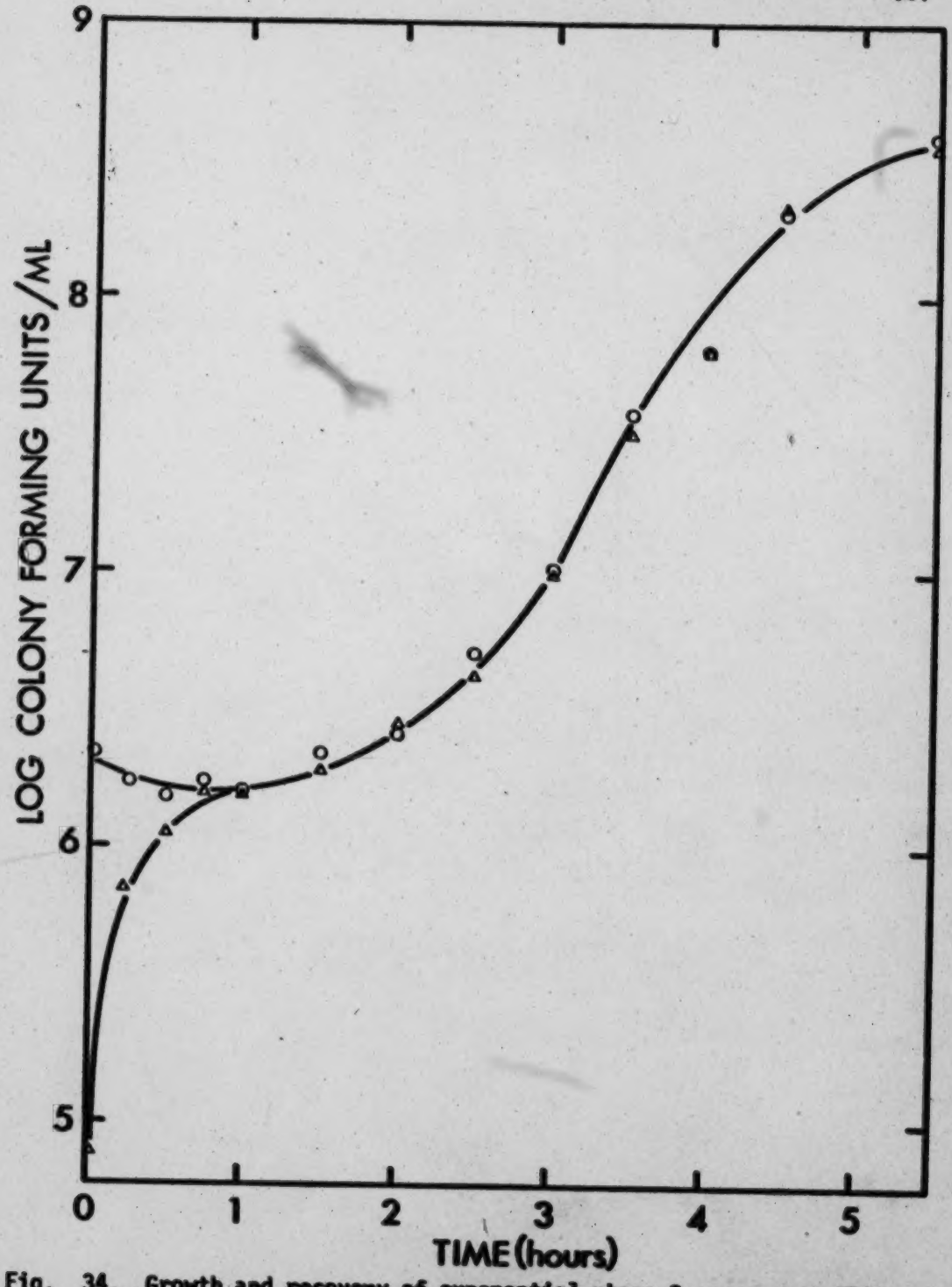

Fig. 34. Growth and recovery of exponential phase S. aureus at 37°C in TSB (pH 7.2) after exposure to 4°C for 6 days. Plating media, o — TSA; Δ — MSA.

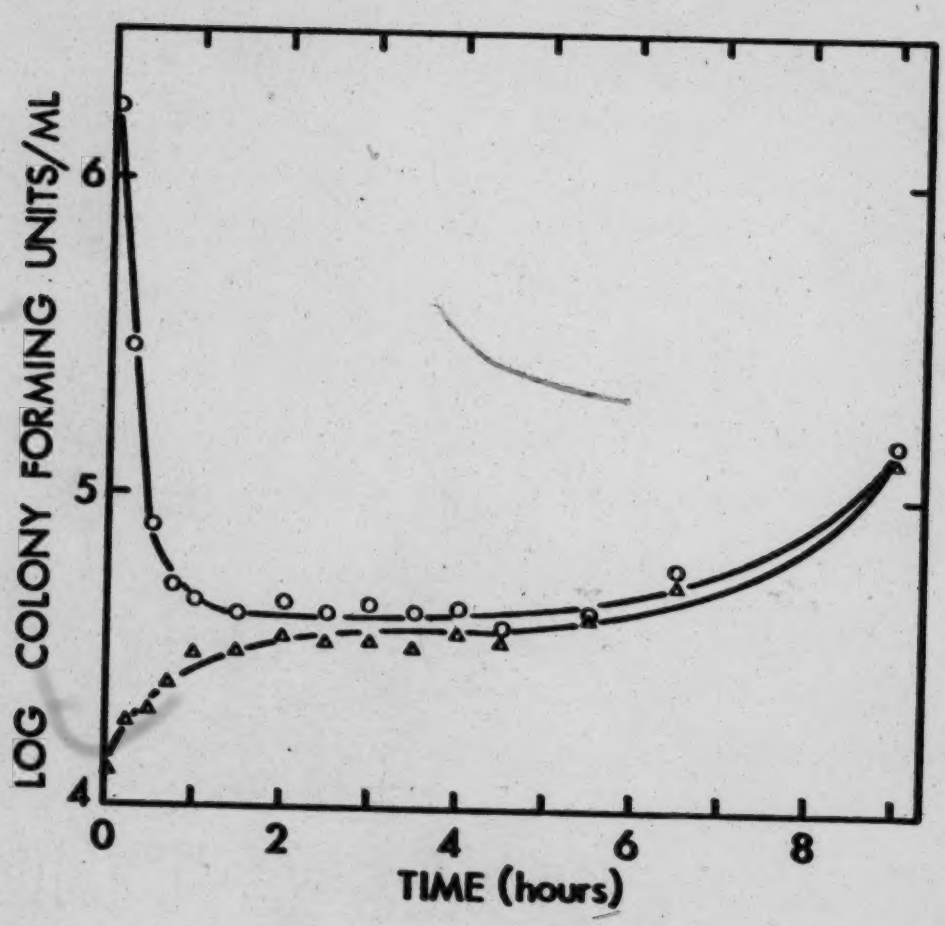

Fig. 35. Growth and recovery of exponential phase S. aureus at 37°C in TSB (pH 5.0) after exposure to 4°C for 6 days. Plating media, o— TSA; Δ—MSA.

At pH 5.0 only a small percentage of the cells recovered their ability to reproduce on MSA (Fig. 35). The injured cells died off and after 1 h incubation only uninjured cells remained viable. Multiplication of the uninjured cells did not commence until after 6 h incubation whereas cells not exposed to the injury temperature commenced growth immediately (Fig. 29).

Recovery of E. coli

Exponential inocula of E. coli injured by storage for 4 days at 4°C in TSB (pH 7.2 or 5.0) recovered rapidly at both pH 7.2 and 5.0 when transferred to 37°C (Figs. 36 and 37). Complete recovery in both instances was demonstrated within 1/2 h incubation and normal growth resumed after an extended lag phase.

Effect of Incubation Temperature on Recovery of S. aureus and E. coli

The effect of incubation temperature on the ability of S. aureus to recover from injury is illustrated in Fig. 38. The culture was injured by exposure of an exponential phase culture to 4°C for 6 days in TSB (pH 7.2). After 24 h incubation in the temperature gradient incubator no recovery of S. aureus could be demonstrated at temperatures below the minimum growth temperature. The extent of recovery increased with increasing incubation temperature and complete recovery occurred at 14 - 15°C. Similar results were obtained with E. coli (Fig. 39). The culture was injured by exposure of an exponential phase culture to 4°C for 4 days in TSB (pH 7.2). Again the organism failed to recover from injury at temperatures below the minimum growth temperature and complete recovery occurred at 11°C.

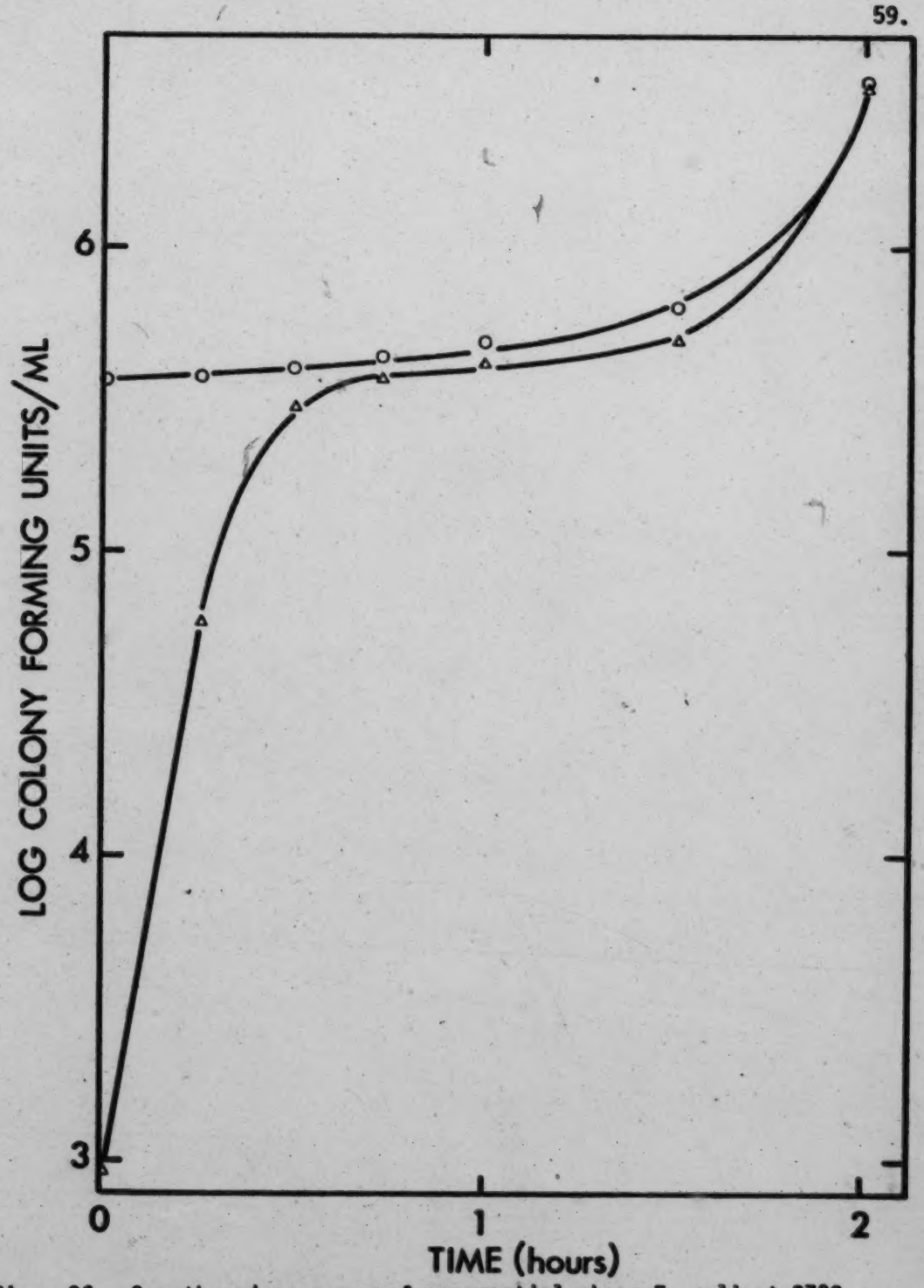

Fig. 36. Growth and recovery of exponential phase E. coli at 37°C in TSB (pH 7.2) after exposure to 4°C for 4 days. Plating media, o — TSA; Δ — VRBA.

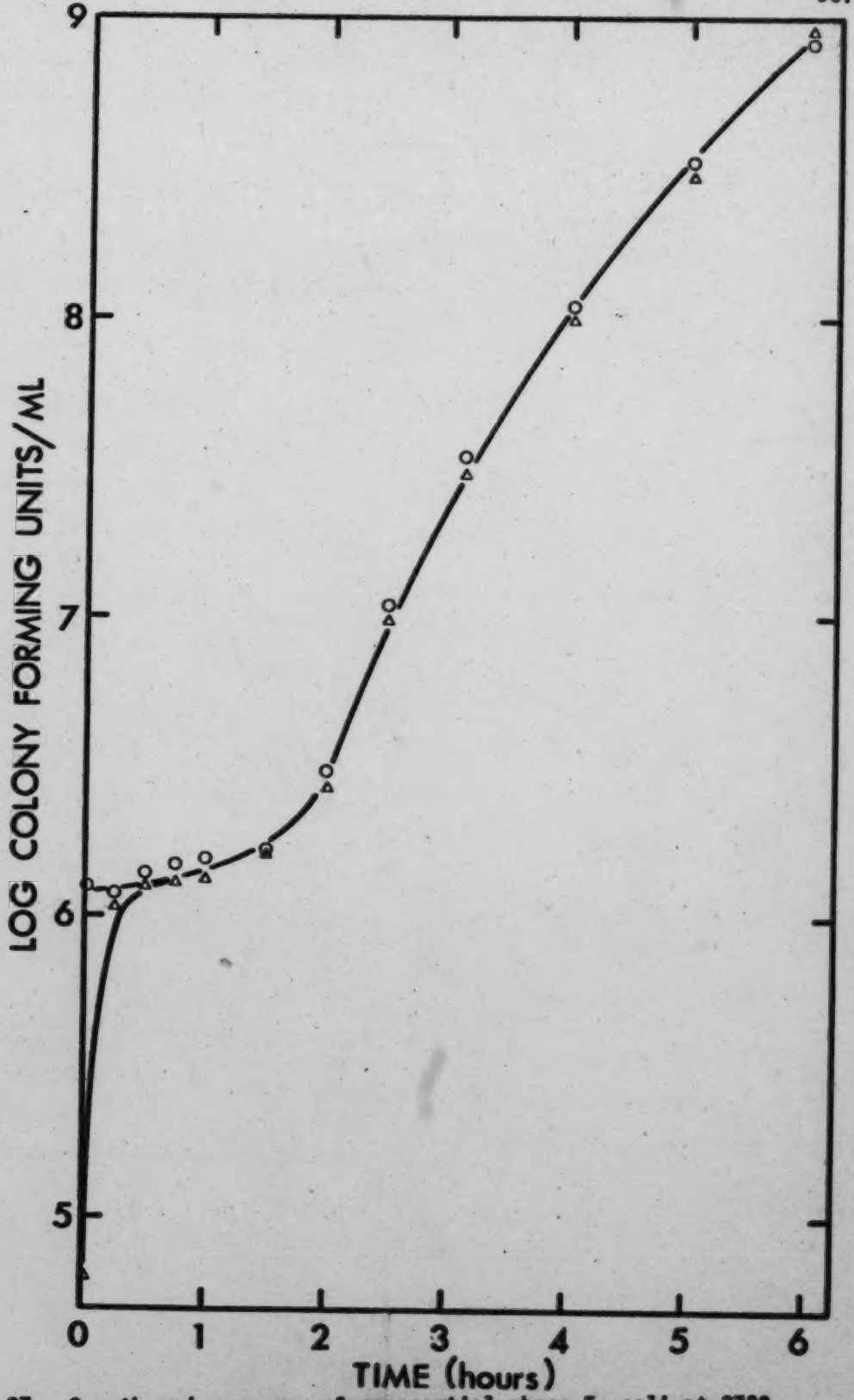

Fig. 37. Growth and recovery of exponential phase E. coli at 37°C in TSB (pH 5.0) after exposure to 4°C for 4 days. Plating media, o —TSA; Δ —VRBA.

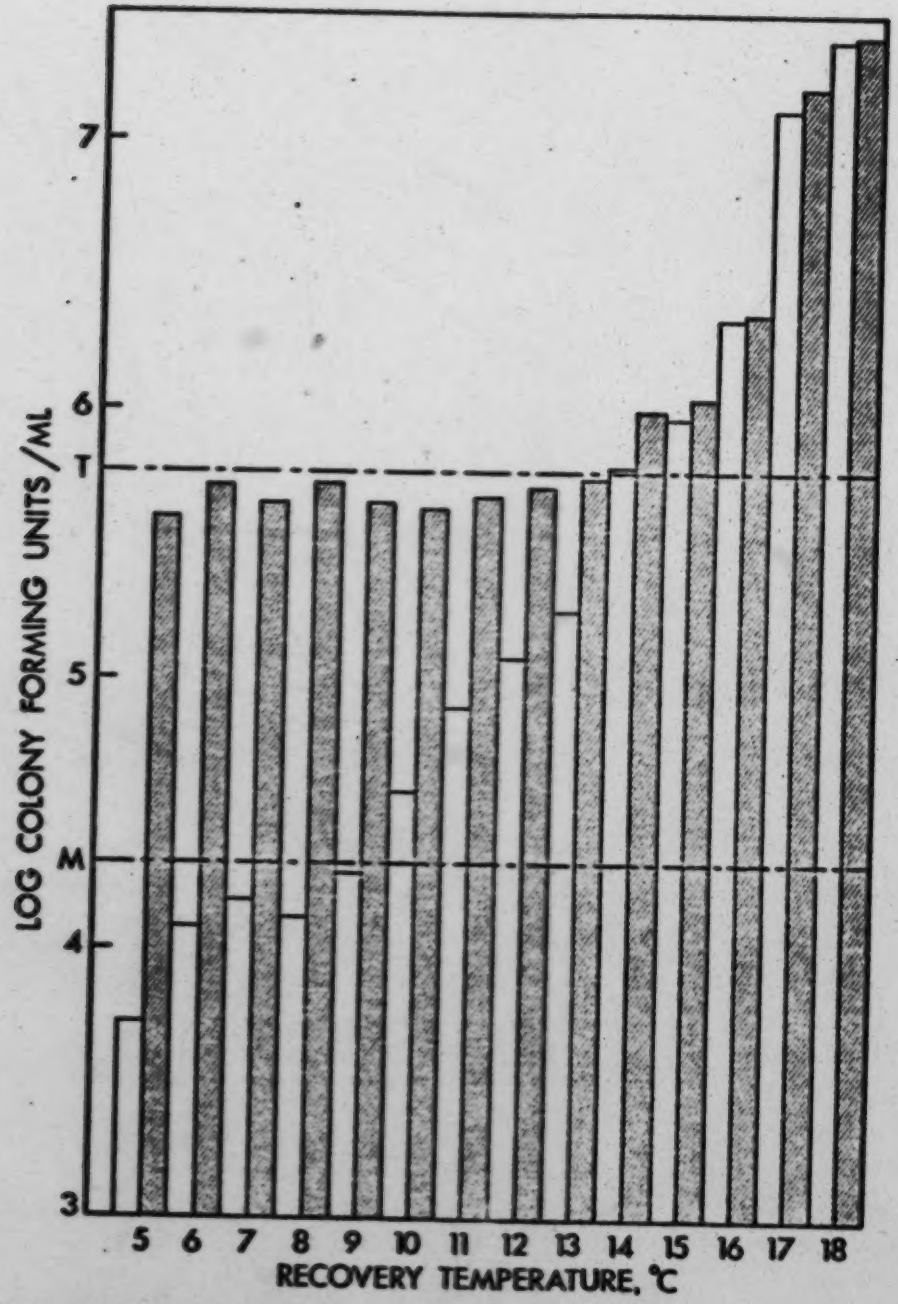

Fig. 38. Effect of incubation temperature on the recovery and growth of injured exponential phase S. aureus in TSB (pH 7.2). Incubation time 24 h. T - initial count on TSA; M - initial count on MSA; shaded - count on TSA; open - count on MSA.

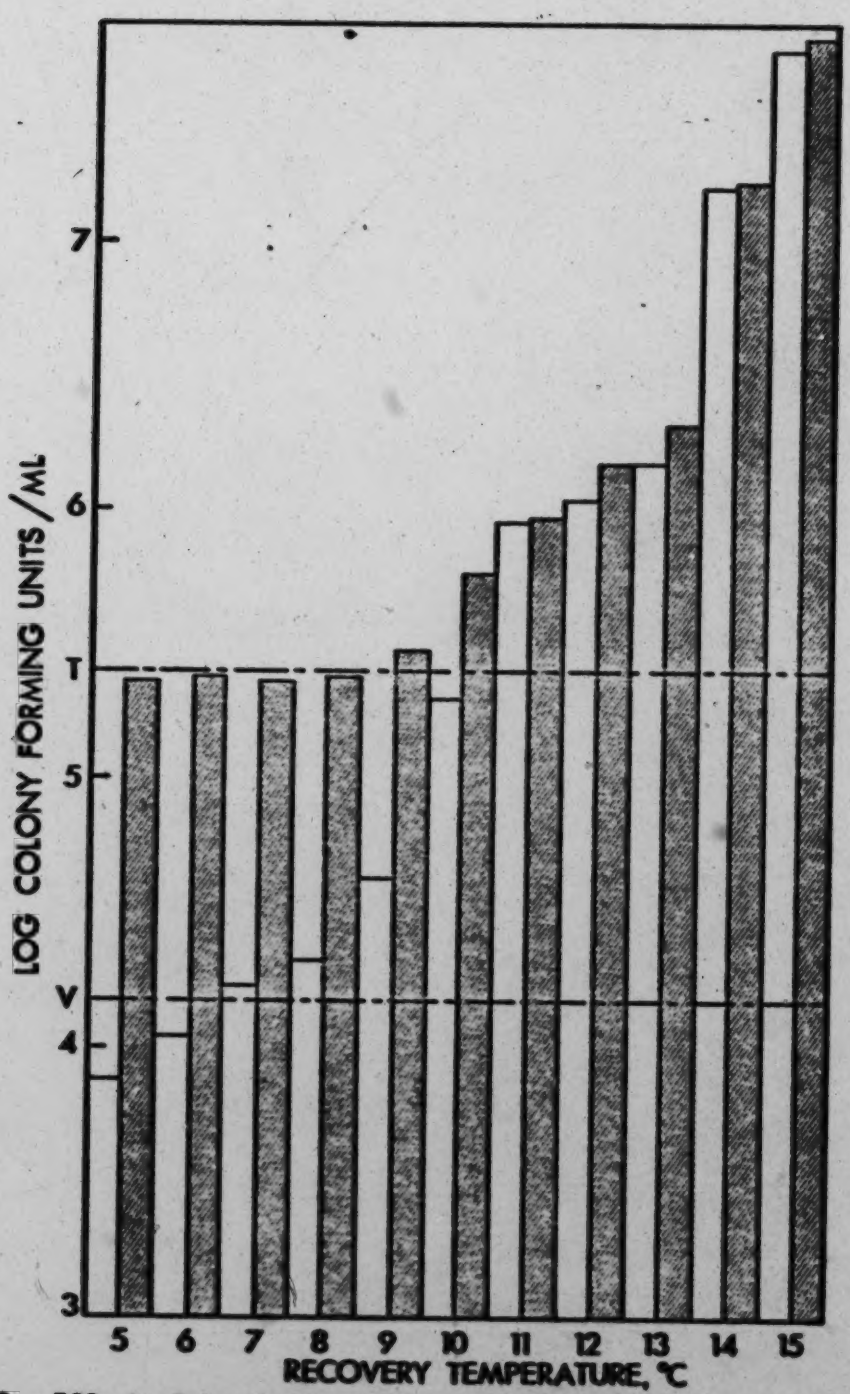

Fig. 39. Effect of incubation temperature on the recovery and growth of injured exponential phase E. coli in TSB (pH 7.2). Incubation time 24 h. T - initial count on TSA; V - initial count on VRBA; shaded - count on TSA; open - count on VRBA.

DISCUSSION AND CONCLUSIONS

This investigation was undertaken primarily to determine the effect of storage at sub-minimal growth temperatures, but above freezing, on the viability of S. aureus, E. coli and Str. faecalis and to ascertain if such exposure would affect the isolation of these organisms on commercially available selective media. In all of the experiments TSA was used as a non-selective control medium. It was found that storage in this temperature range did affect the ability of S. aureus to form colonies on MSA. The capacity of E. coli to form colonies on VRBA was also affected but no modification of the growth of Str. faecalis on KFA could be demonstrated.

Clark et al. (1968) demonstrated the ability of Str. faecalis to recover from injury induced by sub-lethal heating upon incubation in KF Streptococcus broth, indicating that this medium is not particularly inhibitory. It is therefore quite understandable that KF agar would not be sufficiently inhibitory to Str. faecalis after storage at sub-minimal growth temperatures to influence the viable count with respect to the viable count on a non-selective medium.

Busta and Jezeski (1963) and Stiles and Witter (1965) found that the salt tolerance of S. aureus decreased rapidly after exposure to sub-lethal heating. Stiles and Witter further demonstrated a large difference in the count of S. aureus on MSA and a complete nutritional medium after sub-lethal heating. It is interesting to note that the results of the present study demonstrate an analagous decline in the

tolerance of S. aureus to MSA after storage at low temperatures (Figs. 1 and 2). That salt tolerance appears to be the major modification preventing growth on MSA after storage is borne out by the fact that little or no difference between counts on TSA and TPEY could be induced through storage over the experimental temperature range (Figs. 9 and 10).

Both sub-lethal heating and freezing affect the permeability of gram negative organisms. Clark and Ordal (1969) demonstrated that heat injured S. typhimurium displayed an increased sensitivity to bile salts and sodium desoxycholate while Sinskey and Silverman (1970) showed that E. coli became increasingly sensitive to desoxycholate after freeze drying. The storage temperature range used in this investigation caused sufficient damage to E. coli to markedly inhibit its ability to multiply on VRBA which contains bile salts as a selective agent.

The increased sensitivity of exponential phase cultures compared to stationary phase cultures upon exposure to low temperatures has been previously demonstrated with gram negative organisms, and loss of viability at low temperatures correlated with changes in membrane permeability (Strange and Dark 1962, Strange and Ness 1963). Farrell and Rose (1968) presented evidence that insensitivity to "cold-shock" might be associated with increased unsaturation of membrane lipids changing the permeability barrier of the cell. In this study only exponential phase E. coli evinced sensitivity to the temperatures used and exponential phase S. aureus was markedly more prone to injury than stationary phase S. aureus. Since "metabolic injury" may be related to changes in membrane permeability a comparison of the membrane lipids of both S. aureus and E. coli in the exponential and stationary

phases of growth might provide evidence to explain the increased sensitivity of exponential phase culture to "metabolic injury" at low temperatures.

The fact that increasing sodium chloride concentration in the injury menstruum had little or no effect upon the rapidity and extent of injury of S. aureus (Figs. 11, 12, 13) could indicate that the organism must be actively metabolising before the inhibitory effects of sodium chloride at that level become apparent.

An increase in extent of injury as the temperature of incubation was decreased was demonstrated with both E. coli and S. aureus (Figs. 13 - 17, 24 and 25). The cause of temperature induced sensitivity to selective media has not been precisely determined but many proposals have been advanced based mainly on temperature induced degradation of intracellular constituents and physical modification of the membrane. Modifications to membrane permeability allow the leakage of RNA nucleotides (Allwood and Russell 1967, Moss and Speck 1966) and short chain peptides (Moss and Speck 1966). Attempts have been made to correlate leakage with nutritional modifications of the microorganism. It has also been demonstrated that temperature induced increased permeability to metabolic inhibitors could account for increased sensitivity to selective media (Allwood and Russell 1967, Sinskey and Silverman 1971). MacLeod et al. (1966, 1967) suggested that modified membrane permeability allowed the penetration of trace amounts of toxic ions into the cell and proposed that enriched media enhanced the growth of injured cells by chelating the toxic ions. Reduced activity of TCA cycle enzymes after exposure to high temperature has been demonstrated

by Tomlins and Ordal (1971), Bluhm and Ordal (1969) and induced enzyme synthesis has been reported to be impaired at low temperatures (Horiuchi and Novich, 1961).

It would seem most probable that the increased sensitivity to selective media induced by thermal stress cannot be attributed to the modification of any one function of the bacterial cell but rather to the overall damage that temperature extremes induce within the cell. Further work is obviously necessary to ascertain the cellular lesions resulting in metabolic injury during exposure of a microorganism to sub-minimal non freezing environments.

The ability of both S. aureus and E. coli to recover their capacity for growth on the respective selective media used for their enumeration after incubation in TSB at 37°C was demonstrated. Both E. coli and S. aureus exhibited a prolonged lag phase after storage at low temperatures similar to that reported by Jackson and Woodbine (1963) with heat treated S. aureus. Both organisms recovered their ability to grow on selective media during this lag period prior to the initiation of growth. The fact that the count on TSA during this lag period remained relatively constant implies that no reversible impairment of the organisms ability to grow on TSA occurred during storage.

Organisms injured in the exponential phase of growth recovered more rapidly than did those injured in the stationary phase and the extension of the lag phase was less pronounced. It is not possible however, to ascertain whether this difference in recovery period was due to the difference in physiological age of the cells or to the different storage times necessary to induce injury.

E. coli recovered rapidly at both pH 7.2 and 5.0 but S. aureus failed to recover at pH 5.0. The injured cells of S. aureus appeared to be more sensitive to the low pH than the uninjured cells and died off during the lag phase prior to initiation of growth of the uninjured cells. This reaction to low pH could suggest the existence of some factor in the bacterial cell or surrounding menstruum which is adversely affected by low pH, possibly an enzyme system essential for recovery but not for growth.

Both organisms recovered more slowly with decreasing incubation temperature but recovery could not be demonstrated within 24 hours at temperatures below the minimum growth temperature. This precludes the possibility of holding samples at a temperature at which the desired organism would not grow to allow for recovery prior to enumeration on selective media. It is possible that with prolonged periods of incubation that recovery could occur.

In conclusion it has been shown that storage of S. aureus and E. coli at temperatures below minimum growth temperature but above freezing can markedly affect their growth upon certain selective media. The external manifestations of the injury bears a distinct similarity to that induced by harsher treatments such as freezing and thawing, and sub-lethal heating which would be expected to cause more severe damage to the physical structure and metabolic activities of the cell. The rapidity and extent of injury, and recovery from injury was found to be influenced by temperature, pH and physiological age of the organism. The minimum growth temperature was determined under the same cultural conditions used for injury and it was demonstrated that neither organism

was capable of recovery from injury at temperatures below its minimum growth temperature during a 24 h period.

Since E. coli and S. aureus after storage became increasingly sensitive to VRBA and MSA respectively the need for careful selection and screening of media for the isolation of pathogens and indicator organisms from food stored at low temperatures is apparent. The results further indicate that it may be possible to develop resuscitation procedures to maximize recovery of damaged cells from refrigerated foods. Thus samples could be held at 37°C for short periods of time to allow recovery to occur. However, as the recovery period and the duration of the lag phase has been shown to vary, depending upon the physiological age of the cells and of the nature of the medium, resuscitation procedures would need to be subject to very careful evaluation.

REFERENCES

Allwood, M.C. and A.D. Russell. 1967. Mechanism of thermal injury in Staphylococcus aureus. Appl. Microbiol. 15: 1266-1269.

Allwood, M.C. and A.D. Russell. 1968. Thermally induced RNA degradation and leakage of substances from the metabolic pool in Staphylococcus aureus. J. Bact. 95: 345-349.

Arpai, J. 1962. Nonlethal freezing injury to metabolism and motility of Pseudomonas fluorescens and Escherichia coli. Appl. Microbiol. 10: 297-301.

Arpai, J. 1963. Selective effect of freezing as reflected in growth curves. Folia Microbiol. 8: 18-26.

Bluhm, L. and Z.J. Ordal. 1969. Effect of sublethal heat on the metabolic activity of Staphylococcus aureus. J. Bact. 100: 140-150.

Burrows, W. 1968. Textbook of Microbiology. W.B. Saunders Co., Philiadelphia. p. 918.

Busta, F.F. and J.J. Jekeski. 1963. Effect of sodium chloride concentration in an agar medium on the growth of heat shocked Staphylococcus aureus. Appl. Microbiol. 11: 404-407.

Clark, C.W. and Z.J. Ordal. 1969. Thermal injury and recovery of Salmonella typhimurium and its effect on enumeration procedures. Appl. Microbiol. 18: 332-336.

Clark, C.W., D. Witter and Z.J. Ordal. 1968. Thermal injury and recovery of Streptococcus faecalis. Appl. Microbiol. 16: 1764-1769.

Curran, H.R. and F.R. Evans. 1937. The importance of enrichments in the cultivation of bacterial spores previously exposed to lethal agencies. J. Bact. 34: 179-189.

Farrell, J. and L. Campbell. 1969. Thermophilic bacteria and bacteriophages. In A.H. Rose (ed.). Adv. Microbiol. Physiol. Academic Press Inc., New York. 3: 83-109.

Farrell, J. and A.H. Rose. 1967. Temperature effects on microorganisms. In E. Charles and E. Clifton (eds.). Annu. Rev. Microbiol. 21: 101-120.

Farrell, J. and A.H. Rose. 1968. Cold shock in a mesophylic and a psychrophilic Pseudomonad. J. gen. Microbiol. 50: 429-439.

Fay, A.C. 1934. The effect of hypertonic sugar solutions on the thermal resistance of bacteria. J. agric. Res. 48: 453-468.

Friedman, H., P. Lee and A. Rich. 1971. Temperature control of initiation of protein synthesis in Escherichia coli. J. Mol. Biol. 61: 105-121.

Goldstein, A., D.B. Goldstein and L.I. Lowney. 1964. Protein synthesis at 0°C in Escherichia coli. J. Mol. Biol. 9: 213-235.

Gorrill, R.H. and E.M. McNeil. 1960. The effect of cold diluent on the viable count of Pseudomonas fluorescens. J. gen. Microbiol. 22: 437-442.

Gray, R.S., L.D. Witter and Z.J. Ordal. 1973. Characterization of mild thermal stress in Pseudomonas fluorescens and its repair. Appl. Microbiol. 26: 78-85.

Gunderson, M.F. and K.D. Rose. 1948. Survival of bacteria in a pre-cooked fresh-frozen food. Food Research. 13: 254-263.

Harrigan, W.F. and M.G. McCance. 1966. Laboratory Methods in Microbiology. Academic Press, London. p. 21.

Hartsell, S.E. 1951. The longevity and behaviour of pathogenic bacteria in frozen foods. The influence of plating medium. Amer. J. Public Health. 41: 1072-1077.

Horiuchi, T. and A. Novich. 1961. A thermolabile repression system. Cold Spring Harbor Symp. Quant. Biol. 26: 247-248.

Hurst, A., A. Hughes, J.L. Beare-Rogers and D.L. Collins-Thompson. 1973. Physiological studies on the recovery of salt tolerance by Staphylococcus aureus after sublethal heating. J. Bact. 116: 901-907.

Iandolo, J. and Z.J. Ordal. 1966. Repair of thermal injury of Staphylococcus aureus. J. Bact. 91: 134-141.

Ingraham, J.L. 1962. Temperature Relationships. I.C. Gunsalus and R.Y. Stanier (eds.). The Bacteria. Academic Press Inc., New York. 4: 265-296.

Isaacs, M.L. 1930a. Factors which influence tests of bacterial survival. I. The effect of varying periods of incubation of the survival test culture. J. Bact. 20: 161-174.

Isaacs, M.L. 1930b. Factors which influence tests of bacterial survival. II. Effect of pH of the survival test culture on the results obtained with a saprophylic tubercle bacillus. J. Bact. 20: 175-181.

Jackson, H. and M. Woodbine. 1963. The effect of sublethal heat treatment on the growth of Staphylococcus aureus. J. appl. Bact. 26: 152-158.

MacLeod, R.A., L.D. Smith and R. Gelinas. 1966. Metabolic injury to bacteria. I. Effect of freezing and storage on the requirements of Aerobacter aerogenes and Escherichia coli for growth. Can. J. Microbiol. 12: 61-72.

MacLeod, R.A., S.C. Kuo and R. Gelinas. 1967. Metabolic injury to bacteria. II. Metabolic injury induced by distilled water or Cu^{++} in the plating diluent. J. Bact. 93: 961-969.

Meynell, G.G. 1958. The effect of sudden chilling on Escherichia coli. J. gen. Microbiol. 19: 380-389.

Moss, C.W. and M.L. Speck. 1966a. Release of biologically active peptides from Escherichia coli at subzero temperatures. J. Bact. 91: 1105-1111.

Moss, C.W. and M.L. Speck. 1966b. Identification of nutritional components in trypticase responsible for recovery of Escherichia coli injured by freezing. J. Bact. 91: 1098-1104.

Nakamura, M. and D.A. Dawson. 1962. Role of suspending and recovery media in the survival of Shigella sonnei. Appl. Microbiol. 10: 40-43.

Nelson, F.E. 1940. The effect of the new standard milk agar on the plate count of dairy products. J. Bact. 39: 263-272.

Nelson, F.E. 1942. A study of certain factors which influence the apparent heat resistance of bacteria. J. Bact. 44: 389.

Nelson, F.E. 1943a. Factors which influence the growth of heat-treated bacteria. I. Comparison of four agar media. J. Bact. 45: 395-403.

Nelson, F.E. 1943b. Further studies of the effect of the medium on the apparent survival of heat-treated bacteria. J. Bact. 46: 486.

Packer, G.J.K., G.A. Prentice and L.F.L. Clegg. 1973. Design of a temperature gradient incubator. J. appl. Bact. 36: 173-177.

Pasteur, L. 1876. Etudes sur la Biere. In Studies on fermentation 1879. Macmillan and Co. Translation by Frank Faulkner and D. Constable Robb.

Pictet, R. and E. Young. 1884. In W.B. Hugo (ed.). Inhibition and Destruction of the Microbial Cell. Academic Press, London. p. 56.

Postgate, J.R. and J.R. Hunter. 1963. Metabolic injury in frozen bacteria. J. appl. Bact. 26: 405-414.

Ray, B., D.W. Janssen and F.F. Busta. 1972. Characterization of the repair of injury induced by freezing Salmonella anatum. Appl. Microbiol. 23: 803-809.

Ray, B., J.J. Jezeski and F.F. Busta. 1971. Isolation of salmonella from naturally contaminated dried milk products. J. Milk Fd. Tech. 34: 389-428.

Ray, B. and M.L. Speck. 1972a. Metabolic process during the repair of freeze injury in Escherichia coli. Appl. Microbiol. 24: 585-590.

Ray, B. and M.L. Speck. 1972b. Repair of injury induced by freezing Escherichia coli as influenced by recovery medium. Appl. Microbiol. 24: 258-263.

Sato, M., M. Suzuki and H. Takahashi. 1968. Cold shock of germinating Bacillus subtilis spores. Agr. Biol. Chem. 32: 1270-1274.

Sato, M. and H. Takahashi. 1969. Cold Shock of Bacteria. II. Magnesium mediated recovery from cold shock and existance of two critical temperature zones in various bacteria. J. gen. appl. Microbiol. 15: 483-485.

Schmidt-Lorenz, W. 1967. Behaviour of microorganisms at low temperatures. Bull. I.I.R. 2: 1-59.

Sherman, J.M. and W.R. Albus. 1923. Physiological youth in bacteria. J. Bact. 8: 127-139.

Sherman, J.M. and G.M. Cameron. 1934. Lethal environmental factors within the natural range of growth. J. Bact. 27: 341-348.

Sinskey, T.J. and G.J. Silverman. 1970. Characterization of injury incurred by Escherichia coli upon freeze drying. J. Bact. 101: 429-437.

Sogin, S.J. and Z.J. Ordal. 1967. Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury to Staphylococcus aureus. J. Bact. 94: 1082-1087.

Stanier, R., M. Doudoroff and E. Adelberg. 1970. The Microbial World. Prentice Hall, Englewood Cliffs, N.J. p. 354.

Stiles, M.E. and L.D. Witter. 1965. Thermal inactivation, heat injury and recovery of Staphylococcus aureus. J. Dairy Sci. 48:

Stokes, J.L. 1963. In N.E. Gibbons (ed.). Recent Progress in Microbiology. University of Toronto Press, Toronto. 8: 187-192.

Straka, R.P. and J.L. Stokes. 1959. Metabolic injury to bacteria at low temperatures. J. Bact. 78: 181-185.

Strange, R.E. and F.A. Dark. 1962. Effect of chilling on Aerobacter aerogenes in aqueous suspension. J. gen. Microbiol. 29: 719-730.

Strange, R.E. and A.G. Ness. 1963. Effect of chilling on bacteria in aqueous suspension. Nature, Lond. 197: 819.

Strange, R.E. 1964. Effect of magnesium permeability control in chilled bacteria. Nature, Lond. 203: 1304-1305.

Tomlins, R.I. and Z.J. Ordal. 1971. Requirements of Salmonella typhimurium for recovery from thermal injury. J. Bact. 105: 512-518.

Tomlins, R.I., M.D. Pierson and Z.J. Ordal. 1971. Effect of thermal injury on the TCA cycle enzymes of Staphylococcus aureus MF 31 and Salmonella typhimurium 7136. Can. J. Microbiol. 17: 759-765.